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BIOCHEMICAL STUDIES ON A CHLOROPHYLL MUTANT

OF GATEWAY BARLEY

by

PRAFULLACHANDRA VISHNU SANE

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The undersigned certify that they have read, and recommend
to the Faculty of Graduate Studies for acceptance, a thesis
entitled "Biochemical Studies on a Chlorophyll Mutant of Gateway
Barley" submitted by Prafullachandra Vishnu Sane in partial ful-
filment of the requirements for the degree of Doctor of Philosophy.

ABSTRACT

A one gene virescent mutant of Gateway barley was compared with its normal. Changes in amino acids and total soluble sugars in the embryo and endosperm of etiolated seedlings over a 10-day period were studied. The endosperm of the mutant had a lower reserve of protein but the levels of protein and free glycine, which is the nitrogenous precursor of chlorophyll, were similar in the embryos of both lines. Thus the low reserve of nitrogen in the mutant seed was not responsible for its virescent character. The content of total soluble sugars was similar in both lines. Shoots of light grown seedlings of the mutant accumulated considerably more malate than the normal.

The metabolism of glycine-2-^{14}C by 6-day old shoots did not reveal any major differences between the two lines. However, strong evidence for the existence of two separate pools of serine and glutamate in the seedlings was obtained. Acetate-2-^{14}C metabolism by the 6- and 10-day old shoots indicated that the mutant was less efficient in the synthesis of protein particularly at the 6-day stage. The synthesis of leucine from labeled acetate in the mutant was also lower at this stage. Metabolism of leucine-U-^{14}C confirmed the lower rate of protein synthesis in light by the mutant at the 6-day stage. Feeding leucine-U-^{14}C to etiolated shoots in dark showed that protein synthesis in the mutant was impaired in light only.

Based upon the endogenous and labeled amino acid metabolism

ACKNOWLEDGMENTS

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INTRODUCTION

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The research conducted by Maclachlan (1962) and Miller (1965)
on a virescent mutant of Gateway barley showed that the mutant
accumulated chlorophyll at a much lower rate particularly in the early
stages of development. With age the mutant accumulated considerable
chlorophyll but never contained as much as the normal. Their studies
suggested that in the mutant some step of chlorophyll accumulation
was quantitatively affected. The fact that the difference in chloro-
phyll accumulation was greater in the earlier stages of seedling growth
raised the possibility of the differences being due to differences in
food reserves in the seed of the two lines. In order to investigate
this possibility the embryo and the endosperm of etiolated seedlings
up to the 10-day stage were examined for their amino acids, proteins,
and the total soluble sugars which could serve as a source of carbon
and nitrogen for the synthesis of chlorophyll.

In order to determine the step at which the biosynthesis of
chlorophyll was quantitatively affected the metabolism of labeled
acetate, glycine and leucine were followed. The metabolism of acetate
was studied to provide a clue as to which pathway in the mutant was
most affected. Studies on glycine metabolism were expected to tell if
the utilization of this precursor of chlorophyll was different in the
two lines. The object of studying leucine metabolism was to establish
the differences in protein synthesis of two lines in dark and in light.

Studies with inhibitors of protein synthesis in photo-
synthesizing organisms have shown that chlorophyll synthesis is

dependent upon the synthesis of protein (Margulies, 1962; Kirk and Allen, 1965; Gassman and Bogorad, 1965). Gassman and Bogorad (1965) suggested a rapid turnover of δ-aminolevulinic acid (ALA) synthesizing enzymes whereas Kirk and Allen (1965) thought that the protein required for the accumulation of chlorophyll in light was probably the protein moiety of the protochlorophyllide holo-chrome. In view of this it was decided to determine if the synthesis of ALA was limiting in the mutant.

In addition to the major objective of this study which was the comparison of Gateway barley and its mutant, information was obtained relative to the role of glycine and leucine in the synthesis of free amino acids, proteins and other compounds in the barley shoots, as well as the position of free amino acids in the synthesis of proteins in higher plants.

LITERATURE REVIEW

I. Amino Acids

1. Amino acid metabolism of germinating seeds

The germination of a seed starts with the imbibition of water.
The dormant seed gradually undergoes a series of metabolic changes.
In different kinds of seeds the energy for embryo growth is supplied
by various types of food material stored in the endosperm or coty-
ledon. The embryo of the seed which ultimately develops into a plant
needs this nutrition until it becomes an individual photosynthesizing
unit. Studies have been made on the mobilization of food material from
the endosperm to the embryo during germination. Nitrogenous substances
play an important part by acting as energy suppliers and as building
blocks for protein molecules.

Several reviews have appeared on the nitrogen and protein
and amino acid metabolism in plants (Chibnall, 1939; Yemm and Folkes,
1958; McKee, 1958; Webster, 1959; McKee, 1962; Steward and Durzan,
1965; Fowden, 1965). The major changes in nitrogenous substances
during germination of a seed are the breakdown of seed proteins and
nucleic acids, the appearance of free amino acids and amides and the
synthesis of new proteins and nucleic acids in the growing seedling
(Webster, 1959). It has been known since long that a steady decrease
in the protein content during germination of a seed is accompanied by
a concomitant increase in the free amino acids and amides (Chibnall,
1939). Free amino acids account for much of the nitrogen of the

degraded protein. The amide asparagine accounts for a fairly large fraction and the remaining small fraction of protein nitrogen is presumably transformed into glutamine and other nitrogenous compounds. A well accepted scheme for protein metabolism during germination involves degradation of storage proteins to amino acids which are either used for synthesis of seedling protein or broken down to keto acids and ammonia. The keto acids are metabolized to yield energy whereas ammonia is used for synthesis of amino acids and amides.

Brown (1946) compared the changes in nitrogenous substances in embryos of intact grains with those dissected from endosperms two hours after the start of germination. The detached embryos showed a slight decrease in total nitrogen but the embryos attached to the endosperms showed a large increase. In detached embryos a slight increase in the soluble nitrogen at the expense of the embryo proteins was observed. However, in the case of attached embryos both the soluble and insoluble nitrogen (protein nitrogen) increased considerably.

Oota et al. (1953) studied changes in the chemical constituents during germination of bean. A decrease in both the soluble and protein nitrogen was observed in cotyledons whereas in the plumule, radicle, hypocotyl and epicotyl there was an increase. Virtanen et al. (1953) working with pea seeds were able to detect homoserine within 24 hours following germination. Homoserine was not present in the seed protein but the amount of homoserine after 72 hours was greater than any other amino acid. They observed a large amount of free glutamate and considerably smaller amounts of many other amino acids in ungerminated pea seeds. The quantities of all amino acids increased considerably during germination.

The first detailed amino acid analysis of the reserve proteins
of the endosperm and the protoplasmic proteins of the embryo were
undertaken by Folkes and Yemm (1956). Their earlier work (Yemm and
Folkes, 1953) and the work published in 1956 revealed that there was
a considerable difference between the two groups of proteins and it
was concluded that extensive interconversion of amino acids must have
taken place during germination. Later (1958) they undertook studies
of these changes in further detail by examining separately the protein
and soluble fractions of both the endosperm and embryo as they varied
during germination. It was noted that the chief storage proteins of
the endosperm were hordein and hordenin. Earlier (1956) they had
determined the amino acid content of these storage proteins and it
was shown that hordein was rich in glutamic acid, amide and proline;
glutamic acid and proline constituting about 38% of the total nitrogen
of this protein whereas in hordenin glutamic acid and proline con-
stituted only about 18% of the total nitrogen. From their studies
on the changes of these proteins during germination (1958) they con-
cluded that hordein was utilized first. In the endosperm the protein
breakdown was associated with an increase in the non-protein nitrogen
fraction reaching a maximum on the fourth day. The decrease in the
protein nitrogen was far more than the corresponding increase observed
in non-protein nitrogen and it was concluded that there was a trans-
location of simple peptides and amino acids into the growing embryo
at least after 2 days. Most amino acids followed the same general
pattern.

A marked increase in protoplasmic protein was observed in the

case of embryos. There was little change prior to two days but between 2 and 6 days the increase was very rapid. There was practically no change in the level of protoplasmic protein between 8 and 10 days. There was a similar increase in the water-soluble non-protein fraction. The increase occurred chiefly in the basic fraction, peptides and simpler nitrogenous compounds such as amides and amino acids. The basic fraction which was the smallest in the beginning increased rapidly to account for about 35% of the soluble nitrogen. From their studies they drew balance sheets for individual amino acids. From these they observed that the quantities of amide, glutamic acid and proline decreased considerably during germination. The decrease accounted for over 90% of the nitrogen required for new synthesis. A striking increase in α-alanine, arginine, aspartic acid, glycine and lysine along with chlorophyll and other bases was noted. The main changes in the level of amino acid coincided as expected with the period of most active transport of nitrogen from the endosperm to the embryo.

Folkes and Yemm (1956) had earlier concluded from their studies that the endosperm contained in its protein reserves over 70% of the amino acid residues required by the developing embryo. Additional requirements of the embryo included amino acids such as aspartic acid, glycine, lysine and arginine as well as chlorophyll and nitrogen bases. These were synthesized from excess amino acids of the endosperm such as glutamine and proline.

Ferraz (1959) studied qualitative variations of amino acids and organic acids during germination of wheat. He reported an increase in valine, leucine, serine, glutamine and proline during germination.

Karavaeva (1962a) from his studies on dynamics of nitrogen mobiliza-
tion in cotton plants during germination concluded that on germination
disintegration processes prevailed over the synthetic processes in
cotyledons. There was very slight change in the total nitrogen content
of the cotyledons during germination but protein nitrogen decreased
considerably, this decrease being accompanied by an increase in soluble
nitrogen. He observed a high content of amide nitrogen toward the
end of the germination period.

The hydrolysis of endosperm protein and the appearance of amino
acids in the embryo and then in roots and shoots during germination
was confirmed by studies of Sircar and Dastidar (1962). Jiracek et al.
(1962) studying nitrogen metabolism in germinating seeds of pea re-
ported the greatest variation in free amino acids during the first three
days of germination. A rapid increase in free amino acids during this
period was interpreted as a consequence of proteolytic reactions. The
studies of Izawa and Okamoto (1962) on the germinating soybeans
showed that the amount of cotyledonous protein decreased with an increase
in amino and amide nitrogen. A maximum for amino nitrogen was reached
on the fifth day. It was concluded that the amides synthesized in
the cotyledons were translocated to the growing organs.

Boulter and Barber (1963) reported that on germination the
reserve proteins were hydrolyzed to yield at least some free amino
acids that were utilized in the synthesis of new compounds after being
interconverted. They did not observe extensive synthesis of glutamic
acid until six days of growth. The dicarboxylic and basic amino acids
represented a high proportion of the protein nitrogen of ungerminated

seeds. It was suggested that ammonia liberated as a result of utilization of amino acids for respiration was utilized in purine and pyrimidine synthesis. By feeding ^{14}C-arginine during germination they observed that it was degraded by a reversal of the ornithine cycle.

Lawrence and Grant (1963) confirmed the extensive inter-conversion of amino acids during germination with their studies on pea seedlings. The work of Oaks and Beevers (1964) on maize seedlings supports the conclusions drawn earlier by Folkes and Yemm (1958) that nitrogen was transferred to the embryo as amino acids or peptides derived from the hydrolysis of storage protein. They further concluded that the high levels of soluble amino acids normally arriving in the developing embryo from the endosperm restrict their synthesis within the embryo. Oaks (1965) suggested that the demands of the embryo for amino acids regulated the degradation of storage proteins of the endosperm.

Larson and Beevers (1965) working with pea seeds grown in dark and then exposed to light reported high amounts of homoserine in the shoots between the 3rd and 12th days. Aspartic and glutamic acids were also present as major components of the green shoots. Arginine which accounted for over 11% of the free amino acid content of two and one-half day old cotyledons was not detectable in the shoot at any stage. The cotyledon after germination contained notable amounts of proline, glycine and γ-aminobutyric acid, however, these were not found to the same extent in shoots.

McKee (1958) reviewed the work on amide metabolism of seedlings that was done prior to 1958. Yemm (1949) noted that detached barley

leaves, low in sugar, when placed in the dark accumulated a high amount of free amide nitrogen. He further observed that a higher sugar content favoured accumulation of glutamine at the expense of asparagine. McKee (1950) reported that the glutamine content of barley leaves remained consistently low even when large amounts of asparagine synthesis was induced. Meiss (1952) from his experiments on Lupinus albus concluded that in etiolated seedlings the utilization of seed proteins as a substrate for respiration resulted in a striking accumulation of asparagine. About 90% of this asparagine was not further metabolized until a state of acute starvation was reached.

Folkes and Yemm (1958) observed very low levels of glutamine or glutamic acid in embryo of seedlings exposed to light. Karavaeva (1962b) considered asparagine to be the main nitrogen storage compound during growth.

In general, it appears that during germination the reserve proteins are hydrolyzed by proteolytic enzymes and free amino acids are liberated (Mayer and Poljakoff-Mayber, 1963). The fate of liberated amino acids, from the overall changes observed in seed protein and seedling protein, appears to be gradual incorporation into new protein. Very little is known as to what happens to each individual acid. Whether there is extensive interconversion of the carbon skeletons prior to incorporation of amino acids from reserve protein into new protein is not known. This problem is related to the general concept of protein synthesis in higher plants over which there is some controversy. Two hypotheses have been put forth. These are referred to as "the alternative" and the "amino acid" hypothesis (Folkes, 1959).

According to the "alternative" hypothesis the amino acids are the breakdown products of proteins. The work of Gregory and Sen (1937) and Vickery et al.(1940) supported this hypothesis. In recent years further evidence in support of this hypothesis has been adduced by the work of Steward and his associates (Steward et al., 1958; and Pollard and Steward, 1959). According to them new protein is not produced directly from amino acids but by a total synthesis involving carbon skeletons derived from carbohydrate and nitrogen from such donors as ammonia, glutamine or glutamic acid.

According to the "amino acid" hypothesis the formation of amino acids is chiefly the result of nitrogen assimilation and they are the direct precursors of proteins. Evidence for this hypothesis has been reviewed by Wood (1953) and Yemm and Folkes (1958). Folkes and Yemm from their studies on barley (1958) lent further support to this hypothesis. In more recent years this has been confirmed by the work of Cocking and Yemm (1961) and Joy and Folkes (1965).

2. Free amino acids of chlorophyll mutants

Walles (1967) has reviewed in detail the work of Wettstein and Walles on amino acid requiring mutants. There are very few reports on this topic. Fujii and Ono (1960, 1961) working on amino acid content of Einkorn wheat chlorophyll mutants observed that albino and virido albino mutants contained 1.5 to 2 times greater total amino nitrogen than normals whereas in xantha the amount of amino nitrogen present was 0.5 of the normals. It was concluded that the amount of chlorophyll present controlled the amino acid concentration in these mutants.

Veleminsky et al.(1963) investigated the amino acid content in 37 different chlorophyll mutants of Arabidopsis thaliana and 19 mutants of barley. They noted that the amount of amino acids present was 3 to 5 fold greater than in controls. Tyrosine exhibited the greatest variability. Basic amino acids and amides particularly arginine amide showed the greatest increase. They concluded that absence of photosynthesis brought about starvation which was responsible for the observed differences. Maclachlan and Zalik (1963) reported a very high level of free amino acids in the viridis mutant of Gateway barley being investigated. This was further confirmed by Miller (1965) who observed that in light the greatest difference was in the asparagine content. In the dark there was no difference.

Faludi-Daniel and his co-workers (1965) reported studies on free and protein amino acids in normal and chloroplast-mutant corn leaves. They also noted that the free amino acid content of the dark grown leaves was the same but illuminated leaves showed larger amounts of free amino acids in the mutants. A correlation between genetically determined protein synthesis and chloroplast formation was suggested from the differences observed in free and protein amino acid contents of leaves. Perdrizet et al.(1965) reported very high amounts of amino acids in the chemically induced chlorophyll mutants of wheat. In general the levels of amino acids increased from the 2-leaf stage to the heading stage.

Schantz et al.(1966) working with chlorophyll mutants of barley observed higher levels of free amino acids than in leaves of normal.

Examination of the different reports shows that the chlorophyll

mutants always accumulated higher amounts of free amino acids in
light, but there were no differences in the levels of free amino acids
in the dark. In some mutants it has been possible to correct the
chlorophyll deficiency by addition of certain amino acids (Walles,
1967). The chlorophyll mutants usually are unable to form properly
developed chloroplasts. The chloroplasts have been shown to synthesize
protein (Spencer and Wildman, 1964; Spencer, 1965; Goffeau and Brachet,
1965). The accumulation of amino acids in chlorophyll mutants in light may be
due to the inability of their chloroplasts to synthesize protein.

3. Glycine metabolism in plants

Glycine is one of the most important amino acids from the
point of intermediary metabolism. It is one of the earlier products
of photosynthesis (Rabson et al., 1962) and can give rise to several
other amino acids. Glycine is particularly important in green plants
as it provides nitrogen and carbon for the biosynthesis of chlorophylls.
Glycine is also incorporated into purine (Meister, 1965).

The work of Shemin and his associates clearly demonstrated
that glycine supplied nitrogen and carbon for the biosynthesis of heme.
Their work in this respect has been reviewed by a number of workers
(Shemin, 1955; Meister, 1965; Bogorad, 1966). Shemin proposed a
succinate-glycine cycle to show the pathway of glycine metabolism.
The important features of this cycle have been shown schematically in
figure 1. Evidence for the operation for such a scheme has been obtained
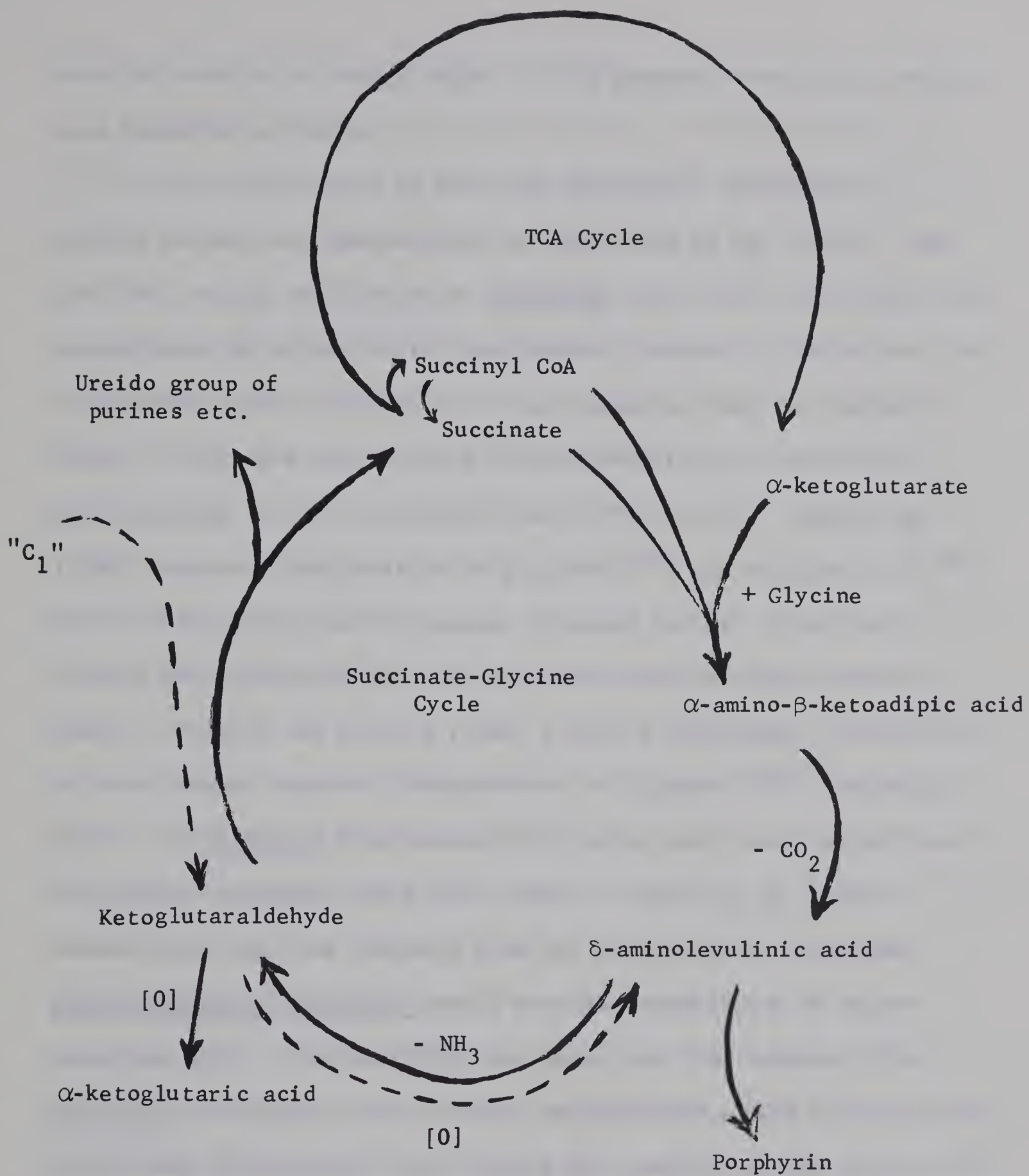
FIG. 1. Succinate-glycine cycle as given by Shemin (1955)

with the studies on animal cells. It is presumed that such a scheme

also operates in plants.

That biosynthesis of heme and chlorophyll proceeds by a

similar pathway was demonstrated by Della Rosa et al. (1953). When

they fed acetate and glycine to Chlorella they found that unlike heme

biosynthesis by animal cells, the carboxyl carbon of glycine was also

incorporated into chlorophylls. They suggested that the carboxyl

carbon of glycine was involved in the formation of a metabolite

participating in the tricarboxylic acid (TCA) cycle. Wang et al.

(1960) reported incorporation of glycine-2-^{14}C and succinate-2,3-^{14}C

into a chloroform soluble fraction of wheat leaves. From their

results they concluded that the succinate-glycine cycle operated in

plants. Perkins and Roberts (1960) studying chlorophyll biosynthesis

in wheat leaves reported incorporation of glycine-2-^{14}C into chloro-

phyll. In Chlorella both carbons of glycine were incorporated into

chlorophyll (Brezeski and Rucker, 1960). Gibson et al. (1961)

demonstrated that the extracts from the photosynthetic organism

Rhodopseudomonas spheroides could catalyze deamination of amino-

levulinic acid. Granick (1954) had shown that the extracts from

Chlorella and spinach could convert aminolevulinic acid into porphyrin.

It has been demonstrated that feeding ALA resulted in the accumulation

of protochlorophyllide in etiolated barley (Granick, 1959, 1961), and

in bean leaves (Sisler and Klein, 1963; Klein and Bogorad, 1964).

All these experiments point out that the pathway for bio-

synthesis of chlorophyll in green plants is similar to the biosynthesis

of heme. Thus one of the ways of glycine utilization in plants is its

incorporation into the chlorophyll molecule.

Glycine is also an important source of carbon and nitrogen for the synthesis of nucleic acids. It has been shown that carboxyl carbon of glycine forms carbon 4 of purine (Sonne et al., 1946; Buchanan et al., 1948), whereas α-carbon and nitrogen of glycine give rise to carbon atom 5 and nitrogen 7 of purine (Shemin and Rittenberg, 1947; Karlsson and Barker, 1949). In wheat seedlings incorporation of labeled glycine into adenine and guanine of ribonucleic acid has been demonstrated (Semenenko, 1949, reported by McKee, 1962). Also Wang and Burris (1965b) showed that glycine-2-^{14}C was incorporated into guanine. It may thus be seen that participation in the synthesis of nucleic acids is another important role of glycine.

Wang and Waygood (1962) working with wheat leaves observed more synthesis of sugars from glycine in light than in darkness. From their isotopic competition studies and intramolecular ^{14}C distribution in the glucose moiety of sucrose they suggested that glucose was formed by the condensation of three carbon compounds. Sinha and Cossins (1964) studied distribution of label from glycine-2-^{14}C in different fractions of castor bean endosperm and carrot tissues. They reported that besides serine and CO_2 radioactivity from glycine-2-^{14}C was present in amino acids, organic acids and sugars. In castor bean endosperm 3% of the total label was incorporated in sugars whereas about 51% of the label was present in amino acids and 7.2% in organic acids. In carrot tissues sugars accounted for only 0.4% of the total ^{14}C incorporated whereas amino acids and organic acids accounted for

27% and 6% of the total label respectively.

Biosynthesis of serine from glycine has been studied extensively in animal tissues (Sakami, 1955). Plants have also been examined in this respect recently. Wilkinson and Davies (1958) showed glycine serine interconversion by using turnip extracts. McConnell and Bilinski (1959) from their experiments with wheat plants suggested that the main pathway for serine biosynthesis involved condensation of carbon 2 of glycine with formate or "one carbon fragment". Davies et al. (1959) reviewed the work done on the subject of glycine serine metabolism and suggested a pathway to reconcile the conflicting reports of glycine and serine labeling during short periods of photosynthesis.

Wang and Burris (1963) noticed that in wheat leaves glycine-2-^{14}C was rapidly converted to serine but glycine formation from serine-1-^{14}C was very low. Their studies confirmed the glyoxylate-serine pathway proposed earlier by Wang and Waygood (1962). These workers later (1965a) confirmed the synthesis of serine from glycine in both light and dark by wheat leaves. Synthesis of glycine from serine, however, was very low. They (1965b) also examined the synthesis of organic acids in light and dark by wheat leaves when fed with glycine-2-^{14}C, serine-1-^{14}C and serine-3-^{14}C.

DeBoiso and Stoppani (1963) obtained direct evidence indicating condensation of glycine with C_1 compound as the mechanism for the bio-synthesis of serine in baker's yeast and Torulopsis utilis. Sinha and Cossins (1964) from their work with carrot tissues and castor bean endosperm demonstrated glycine serine interconversion. The results

suggested that glycine was converted to glyoxylate which on decarboxylation gave rise to C_3 of serine. They also suggested that glycine underwent degradation and resynthesis. They later (Cossins and Sinha, 1966) investigated in detail the interconversion of glycine and serine in plant tissues. Studies involving several plant tissue extracts revealed that serine formation was favoured. From their results they concluded that glycine was cleaved directly to yield α-carbon which became β-carbon of serine. The C_1 unit was transferred via N^5, N^{10}-methylenetetrahydrofolate. It was further concluded that extracts from higher plant tissues synthesized serine from glycine without the formation of glyoxylate or formate as intermediates. In this connection it may be mentioned that Sanadi and Bennett (1960) working with mitochondrial fragments from chicken liver did not observe significant decreases in the incorporation of ^{14}C from glycine to serine by the addition of glyoxylic acid and formaldehyde to the system. They suggested that the intermediates in the conversion were tightly bound to the enzyme complex and were not readily dissociated.

II. Organic Acid Metabolism of Germinating Seeds

The work on organic acid metabolism has been reviewed by Thimann and Bonner (1950), Fowden and Moses (1960), Zelitch (1964), Ranson (1965), Beevers et al. (1966). The review by Beevers et al. provides a thorough discussion of all aspects of organic acid metabolism in plants. During germination of cereal and legume seeds the changes in organic acids have been studied by a few workers and a brief summary of these studies is given below.

Fowden and Moses (1960) discussed in detail the work of
Taufel and his co-workers who examined the changes in citric acid
content of rye, barley, pea, rape and linseed during germination. In
barley they noted a 100% increase in citric acid at the 5-day stage
as compared to citric acid content of original seeds. The work of
Soldatenkov and his associates (reported by Fowden and Moses, 1960)
showed that the shoots of wheat, rye and maize contained much higher
amounts of di- and tri-carboxylic acids than their content in seeds.

Studies on the changes of α-ketoglutaric, pyruvic and glyoxylic
acids in germinating wheat seeds showed that these acids increased
rapidly after germination and later decreased (Krupka and Towers, 1958).

Ivanovskaya (1960) working with corn observed that citric acid
content of leaves varied at different stages of development. The roots
contained lower amounts of citric acid than the leaves. The work of
Grineva (1961) showed that anaerobiosis resulted in the inhibition of
organic acid synthesis. This inhibition was connected with glycolysis
and the Kreb's cycle. Yamamoto (1961) working with bean cotyledons
confirmed that the TCA cycle was not operative in this tissue but the
glyoxylate cycle was present. The evidence for the operation of
glyoxylate and TCA cycle and their role in plants has been reviewed
in detail by Beevers et al. (1966).

Shramm and Piatkowska (1961) separated the organic acids of
broad bean with paper chromatography. All the tissues of this plant
examined contained citric and malic and small amounts of succinic
and fumaric acids. Citric acid predominated in roots.

Duperon (1961) working with Phaseolus vulgaris showed that

β-carboxylation was responsible for the synthesis of malic acid in the dark. Later (1963) he confirmed that β-carboxylation of phosphoenolpyruvate yielded oxaloacetic acid which was then reduced to form malic acid. He suggested that malonic acid was produced from acetyl CoA by carboxylation. Wall et al. (1961) studied the organic acids of barley. They noted that malic, acetic, succinic, fumaric, malonic, α-ketoglutaric, lactic, citric, aconitic and pyrrolidone carboxylic acids accounted for most of the organic acids. Some phenolic acids were also present. Sabala (1962) examined seeds of different types for citric, malic and total organic acid contents and noticed that viable seeds contained greater amounts of these acids than non-viable seeds. This was in agreement with the observation of Taufel and Pohloudek-Fabini (reported by Fowden and Moses, 1960) who showed that the germinating ability of seed with higher citric acid content was higher than the seeds with lower citric acid content. Soldatenkov (1962) in a review on organic acids of higher plants questioned the universal application of TCA cycle on some grounds. He suggested that malic, citric and other di- and tri-carboxylic acids could originate from saccharinic and saccharonic acids.

Miryakubova (1963) studying the organic acids of corn noted the presence of citric, malic, aconitic and some unidentified acids. Synthesis of di- and tri-carboxylic was particularly high in young plants.

III Chlorophyll

1. Chlorophyll biosynthesis

Several reviews have appeared recently (Granick, 1951;
Godnev and Shlyk, 1956, 1961; Gibson et al., 1961; Granick and
Mauzerall, 1961; Smith and French, 1963; Lascelles, 1965; Bogorad,
1965a, b, 1966; Marks, 1966; Granick, 1967) which discussed in
detail the steps involved in the biosynthesis of heme and chlorophyll
starting from glycine and succinate. A well documented scheme for
chlorophyll biosynthesis given by Bogorad (1965a) has been reproduced
schematically in figure 2. The biosynthetic chain up to proto-
porphyrin is the same for heme and chlorophyll biosynthesis. From
here, one pathway involving incorporation of Mg leads to chlorophyll
and another involving incorporation of Fe leads to heme synthesis.
The enzyme systems catalyzing synthesis of iron protoporphyrins have
been found in many organisms (Marks, 1966). However, similar systems
catalyzing synthesis of Mg-protoporphyrin have not been observed in
plants and other photosynthetic organisms. The synthesis of heme
by the mitochondria of animal cells has been shown, however, the
mitochondria isolated from plant cells have yet to be investigated
in this respect (Granick, 1967). In plants, chloroplasts are
organelles that may be capable of synthesizing chlorophyll and heme.
Conversion of ALA into protoporphyrin by Euglena chloroplasts has been
demonstrated by studies of Carrel and Kahn (1964). Recently Jones
(1967) has shown that isolated spinach chloroplasts purified on
discontinuous gradients can synthesize heme from protoporphyrin IX,
mesoporphyrin IX and deuteroporphyrin IX in the presence of ferrous

Succinyl CoA + Glycine

$-CO_2$ | ALA synthetase
Pyridoxal phosphate

δ-Aminolevulinic acid

$-2H_2O$ | ALA dehydrase

Porphobilinogen

UROGEN I synthetase | UROGEN I synthetase
+
UROGEN III cosynthetase

Uroporphyrinogen I | Uroporphyrinogen III

-4 COOH | -4 COOH
UROGEN decarboxylase

Coproporphyrinogen I | Coproporphyrinogen III

COPROGEN
Oxidative decarboxylase

Protoporphyrinogen IX

Ferrochelatase
Fe protoporphyrin IX ← ———————— Protoporphyrin IX
+ Fe^{++}

+ Mg

Cytochromes, peroxidase, | Mg protoporphyrin IX
catalase, hemoglobin, | +
phycobilins | S-adenosyl | Mg proto methyl esterase
methionine

Mg protoporphyrin monomethyl ester

?
?

Mg 2,4-divinyl pheoporphyrin a_5

Chlorophyll b ← — — — —Chlorophyllide b
+ Phytol | ?
? | + 2H
? / |
Chlorophyllase (?) | + Phytol
Chl. a ← ———————— Chlorophyllide a ← —— Protochlorophyllide a ——→ Protochloro-
+ Phytol phyll a
\ ? + 2H
\ [Transformation may be of
protochlorophyllide-holochrome only]
Bacteriochlorophyll

FIG. 2. Scheme for the biosynthesis of
chlorophyll as given by Bogorad (1965a)

ions and under reduced conditions. It has not yet been shown whether plastids can synthesize ALA and succinyl CoA.

Besides glycine and succinate, incorporation of carbon atoms of proline into chlorophyll has been reported. Duranton and Maille (1961) studying the metabolism of proline in Jerusalem artichoke reported incorporation of label into chlorophyll _a_, _b_ and xanthophylls and carotenoids, 6 to 8 hours after administration of proline-U-^{14}C. The amount of label in the chlorophyll was approximately 1% of the total utilized by plants. Later (1962) they reported appreciable incorporation of ^{14}C from proline into the tetrapyrrole nucleus of chlorophylls _a_ and _b_. Duranton and Schantz extended these investigations to Chlorella pyrenoidosa and reported (1965) that proline ^{14}C was incorporated into the tetrapyrrole group of the chlorophyll. Further studies on the incorporation of proline into chlorophyll of Chlorella revealed that the carbon atoms were incorporated more readily into the tetrapyrrole nucleus of the pigment than into the phytol chain (Schantz and Moissenot, 1966). The radioactivity present in the tetrapyrrole nucleus was of the order of 91-96%. The feeding studies with glutamic acid-U-^{14}C showed that 80% of the activity was present in the tetrapyrrole nucleus and about 20% in phytol. They explained that the weak labelling in the phytol chain might have been the result of a preferential incorporation of the carbon into the Shemin succinate glycine cycle or an isotopic dilution via acetate. In this connection it is interesting to note that Marks and Bogorad (1960) had reported that in Serratia marcescens proline or a closely related derivative of proline was a precursor of prodigiosin; 5-aminolevulinic

acid was not incorporated into this red pigment.

Although glycine and succinate via ALA may be the main path of pyrrole synthesis there is a considerable similarity between the proline and pyrrole structure and an alternate but minor pathway from proline to pyrrole cannot be ruled out completely.

2. Control mechanisms of chlorophyll synthesis

Several attempts to investigate control mechanisms in the biosynthesis of heme and chlorophyll have revealed that the situation is very complex. Lascelles (1965), Bogorad (1965a, b, 1966), Marks (1966) and Granick (1967) have reviewed and discussed evidence with regard to control mechanisms of heme and chlorophyll synthesis in animals and higher plants. In the following section the relation between protein synthesis and chlorophyll accumulation has been emphasized.

Rubin (1957) observed that streptomycin induced the development of albinism in Hordeum vulgare. From his studies he concluded that chlorophyll synthesis was connected with the cytochrome system and especially cytochrome oxidase.

Wang et al. (1960) observed that addition of benzimidazole to wheat seedlings stimulated incorporation of ^{14}C from glycine or succinic acid into chlorophyll.

Hudock et al. (1964) from their studies on a mutant strain of Chlamydomonas reinhardi showed that during earlier stages of re-greening, synthesis of protein was essential for the development of well organized chloroplasts and a high rate of photosynthesis.

Kirk and Allen (1965) studied the effect of actidione on the synthesis of chloroplast pigments in Euglena. They clearly showed that chlorophyll a synthesis was completely dependent upon the synthesis of protein. They thought that chlorophyll a synthesis required the formation of some protein in stoichiometric rather than catalytic amounts. The protein moiety of protochlorophyllide holo-chrome or the protein in the chloroplast lamellae to which the chloro-phyll is bound could constitute the protein in question. Another explanation could be that one or more enzymes involved in chlorophyll a biosynthesis underwent rapid turnover. According to Gassman and Bogorad (1965) production of chlorophyll at a constant rate following the lag phase was dependent upon continuous protein synthesis. They postulated a rapid turnover of the enzymes of δ-aminolevulinate synthesis.

Margulies (1967) reported inhibition of the decrease in lag phase of chlorophyll accumulation by chloramphenicol. It was proposed that chloramphenicol inhibited protein synthesis required for chloro-phyll accumulation. From the results obtained it was not possible to determine whether the synthesis of protein was required for production of a protein needed in stoichiometric amounts as suggested by Kirk and Allen (1965) or for formation of enzymes needed for chlorophyll biosynthesis.

Kasemir and Mohr (1967) observed that actinomycin D did not inhibit the synthesis of protochlorophyll but the de novo synthesis of some specific structural protein required for accumulation and maintenance of chlorophyll a in plastids was inhibited.

Troxler and Bogorad (1967) working with _Cyanidium caldarium_ reported that chloramphenicol inhibited phycocyanin accumulation in illuminated cells of this alga. It was concluded that protein synthesis was necessary for the formation of this pigment. It was also demonstrated that for the synthesis of ALA or its precursor light was essential. The inability of the cells treated with inhibitor to utilize ALA in dark was interpreted to mean that terminal steps in the synthesis of phycobilin from ALA were enzymatic.

Linnane and Stewart (1967) presented evidence regarding the inhibition of chlorophyll formation in _Euglena_ by the antibiotics chloramphenicol, tetracycline and lincomycine which inhibit protein synthesis in bacteria and mitochondria.

Banerji and Laloraya (1967) reported inhibition of chlorophyll _a_ and _b_ formation in isolated pumpkin cotyledons. The inhibition appeared to be due to interference with protochlorophyll synthesis and its conversion to chlorophyll. The greater inhibition of chlorophyll _a_ formation than chlorophyll _b_ formation by chloramphenicol was interpreted as evidence for two divergent pathways for the synthesis of chlorophylls _a_ and _b_.

Gassman and Bogorad (1967a) from their work with protein and nucleic acid inhibitors concluded that chlorophyll synthesis in bean leaves was regulated by light by controlling the availability of ALA. In a later (1967b) communication they reported that the regeneration of protochlorophyllide resulted from the synthesis of RNA and enzymes needed for the synthesis of ALA.

It would thus appear that the biosynthesis of chlorophyll in

green cells needs synthesis of some kind of protein. There is also evidence to show that light induces synthesis of a specific RNA which is necessary for chloroplast protein and chlorophyll formation (Molotkovskii and Moryakova, 1963).

Bogorad and Jacobson (1964) from their observation on inhibition of greening of etiolated leaves by actinomycin D concluded that DNA dependent synthesis of RNA was required for chloroplast formation from plastids and light appeared to induce RNA synthesis. An interrelation between chlorophyll synthesis and accumulation of nucleic acids has been shown by Lebedev and Litvinenko (1966). Beridze et al. (1966) obtained evidence with respect to the participation of nucleic acids in chlorophyll synthesis. Their work with inhibitors of RNA synthesis, actinomycin D, 2-thiouracil and 8-azaguanidine, has demonstrated that the synthesis of light induced RNA was obligatory for chlorophyll formation.

The inhibitory action of chloramphenicol in cells containing chloroplasts is largely confined to the protein synthesis in chloroplasts. Smillie et al. (1963) have shown that the antibiotic concentration required for inhibition of chloroplast protein synthesis during greening of Euglena had little effect on the synthesis of certain cytoplasmic proteins. The cytoplasmic protein synthesizing systems isolated from photosynthetic cells were comparatively insensitive to low concentrations of chloramphenicol (Eisenstadt and Brawerman, 1964) whereas systems isolated from chloroplasts were sensitive (Goffeau and Brachet, 1965; Spencer, 1965). Anderson and Smillie (1966) investigated this fact and showed that chloramphenicol

was more strongly bound by chloroplast ribosomes than by neighboring
cytoplasmic ribosomes. They observed that in the case of pea leaves
as much as 22 mµg of chloramphenical was bound per mg of RNA of
chloroplasts whereas only 7 mµg of chloramphenical was bound per mg
of RNA of cytoplasm. Comparable values in case of wheat were
28 mµg of chloramphenical per mg of RNA of chloroplasts as against
11 in the case of cytoplasm. Aaronson et al.(1967) have recently
confirmed the observation of a difference between in vivo sensitivities
of cytoplasmic and organelle protein synthesis to chloramphenicol.
In Euglena they observed that chloramphenical and DL-ethionine inhibited
in vivo protein synthesis more in chloroplasts than in cytoplasm. On
the basis of this it may be pointed out that the protein synthesis
required for the accumulation of chlorophyll as shown by inhibitory
studies with chloramphenical may probably be the protein synthesized
by chloroplasts. Whether this protein is an enzyme required in the
biosynthesis of chlorophyll or it is a protein needed in stoichiometric
amounts is not known.

In chlorophyll mutants with a few exceptions the biosynthetic
chain of chlorophyll is either on or off. Since the intermediates do
not accumulate an important control point may be the functioning of
ALA synthetase (Bogorad, 1965a). Granick (1959) reported that incubation
of etiolated leaves with ALA resulted in an accumulation of larger
amounts of protochlorophyllide. This suggested that the activity of
ALA synthetase was limiting. Lascelles (1965) after taking into
account the work of different people suggested that the key enzyme
probably was ALA synthetase. Kaler and Podchufarova (1965) from

their work on etiolated barley shoots concluded that the controlling

factor in the biosynthesis of protochlorophyllide was either

succinyl CoA synthetase or ALA synthetase. Burnham and Lascelles (1963)

had earlier suggested that feed back inhibition of ALA synthetase

by hemin might be one means of controlling porphyrin formation. On

the basis of studies made on bacterial and animal cells by several

investigators Granick (1967) suggested that the control of heme

biosynthesis was by heme repression of the synthesis of ALA synthetase

or inhibition of its activity. Granick and his associates (Levere et al.,

1967) have observed that certain steroids stimulated 2- to 3-fold the

early formation of hemoglobin in erythroid cells of chick blastoderm

cultured in vitro. They proposed an attractive hypothesis on the

control of heme formation involving a repressor-operator mechanism

that controls the synthesis of the limiting enzyme ALA synthetase.

This mechanism, according to them, is derepressed by an unknown

physiologic derepressor causing enhanced porphyrin synthesis. It

is not unlikely that in the plants a similar mechanism may operate,

the derepressor being released only when plants are exposed to light

thereby causing increased synthesis of ALA synthetase and finally

chlorophyll.

Lascelles (1965) pointed out that chlorophyll accumulation

may be controlled by the Mg vinyl pheoporphyrin-a_5 (MgVP)

holochrome exerting some form of control on the action of the enzymes

leading to its synthesis. Granick (1967) while discussing control

mechanisms suggested that in light-requiring algae and higher plants

MgVP reduction might be the step at which control was exerted. This

step according to him might be related to the temporary removal
of a block in ALA synthetase.

Intracellular localization or compartmentation of enzymes of
porphyrin biosynthesis was suggested as another control mechanism of
porphyrin biosynthesis by Sano and Granick (1961).

Bogorad (1965b) has discussed the evidence in respect of
involvement of phytochrome in chlorophyll production. Recent
reports by Shlyk et al.(1966) and Kasemir and Mohr (1967) further
confirm the role of phytochrome in the control of chlorophyll
formation.

It appears that the control mechanisms in the biosynthesis
of heme or chlorophyll are still obscure. More information is
needed to understand clearly these control mechanisms.

MATERIALS AND METHODS

I. Plant Material

A one gene mutant of Gateway barley was used for conducting the studies. The mutant has been described as virescens by Maclachlan and Zalik (1963) and was designated as yv_2 mutant located on chromosome I (Walker et al., 1963). The seedlings of the mutant on emergence were pale but gradually became greener as they grew older. The mutant seedlings, however, never contained as much chlorophyll as the normal seedlings.

The parent variety Gateway (normal) was used for the purpose of comparison. The seeds of both the normal and the mutant were obtained by growing the seedlings under glass house conditions at the same time.

II. Growing Seedlings

To study the amino acid content and total soluble sugars of the seeds, the seeds of the normal and the mutant were soaked in distilled water for 2 hours. The lemma and palea were removed and the seeds were divided into endosperm and embryo (containing scutellum).

The seedlings were grown either in dark or in light for the required period of time. The seeds were planted on wet vermiculite and covered with a thin layer of vermiculite. The trays were kept in a germinating cabinet at 23° and distilled water was added every other day. Light grown seedlings were subjected to a cycle of 8 hours dark and 16 hours light at an intensity of 200 ft-c.

Etiolated seedlings were harvested 2, 4, 6, 8 and 10 days after planting to determine amino acid content and total soluble sugars. The weight of each individual seed was taken and a record kept of its position in the tray. Only seedlings that developed uniformly were selected and removed with their roots intact. They were then washed thoroughly with water and separated immediately into the endosperm and embryo (including scutellum, roots and shoots) after removing the lemma and palea. There were two replications of 50 seedlings each.

The organic acid determinations were made on shoots and root plus endosperm portions of seedlings grown in light. The determinations for 0- and 2-day samples were made on whole seeds since there was not much development of the shoot in these cases.

III. Feeding of Labeled Compounds

Twenty-five shoots from seedlings grown either in dark or light for 6 or 10 days were excised 1 cm above the vermiculite. These were placed in vials containing the label. They were incubated at room temperature for the desired period either in dark or in light (200 ft-c). The shoots usually took up all the radioactive solution in the first 40 minutes after which 0.5 ml of distilled water was added to the vials. At the end of the feeding period the shoots were washed thoroughly prior to further processing.

The radioactive compounds used were:

(1) Acetate-2-^{14}C (as sodium acetate, specific activity 25.5 mc/mmole)

(2) Glycine-2-^{14}C (specific activity 2.89 mc/mmole)

(3) Leucine-U-^{14}C (specific activity 251.4 mc/mmole)

(4) δ-aminolevulinic acid-4-^{14}C (specific activity 24.5 mc/mmole)

Compounds 1 and 4 were obtained from CALBIOCHEM and compounds 2 and 3 from New England Nuclear Corporation.

IV. Extraction and Sample Preparation

Except for the studies involving pigment analysis the plant materials after being cut into pieces of approximately 1 cm length were plunged into 80% boiling ethanol to inactivate the enzymes. The samples were extracted with 80% ethanol by grinding with a mortar and a pestle. The ground material was centrifuged with approximately 35 ml of 80% ethanol for 20 minutes at 20,000 g. The supernatant was decanted and the residue resuspended for 5 minutes in about 35 ml of 80% ethanol. It was again centrifuged and the procedure repeated 2 more times. Tests for the presence of amino acids and sugars conducted on the supernatant of the 4th extraction showed that all the free amino acids and soluble sugars were extracted completely by the 3rd extraction. The supernatants from all the 4 extractions were pooled and evaporated to dryness under vacuum at 40°. The extraction of the material with 80% ethanol separated the soluble material from the pellet.

The soluble fraction was made up to 40 ml with distilled water and was extracted 4 times with 25 ml of diethyl ether. The ether extracts were pooled for measurement of radioactivity in studies involving labeled compounds. In case of studies involving organic acid determinations the soluble fraction was extracted 4 times with chloroform instead of diethyl ether. The lipid free soluble material was evaporated to dryness under vacuum at 40°. The residue was made up in 5 or 10 ml of distilled water depending upon the plant material used.

The pellet fraction was extracted 3 times with diethyl ether. The extracts were pooled to measure radioactivity in studies involving labeled compounds.

Fractionation of the soluble material into amino acids, soluble sugars and organic acids:

The methods used were similar to those used by Canvin and Beevers (1961) and Cossins and Beevers (1963). Separation of amino acids from organic acids and sugars was achieved by placing a 2 ml aliquot on a 1 x 5 cm column of AG 50 W-X 8, 200-400 mesh, hydrogen form. The column was washed with 40 ml of distilled water to elute sugars and organic acids. The amino acids which were held on the resin column were then eluted with 40 ml of 2N ammonium hydroxide. Each of the fractions was evaporated under vacuum at 40°. A similar procedure was adopted to separate amino acids from other substances in protein hydrolyzates for studies involving labeled compounds.

Separation of organic acids from sugars was accomplished by using an ion exchange resin in the formate form. Anion exchange resin AG 1- X 10, chloride form, 200-400 mesh was obtained from CALBIOCHEM. In order to convert this resin into formate form the resin was washed with 1 M sodium formate until the effluent gave a negative test for chloride. It was then washed with 0.1 N formic acid (2 volumes of formic acid to 1 volume of resin). The resin was finally washed with distilled water until the effluent was neutral. The sample containing organic acids and sugars was placed on a 1 x 5 cm formate column and washed with 40 ml distilled water to elute the sugars. The organic acids held on the column were then eluted with 40 ml of 4 N formic acid.

Each of the fractions was dried under vacuum at 40° and made up to a desired volume in distilled demineralized water.

Protein amino acids: The lipid free pellet was dried under vacuum at 60° and either a fraction or the whole pellet was re-fluxed with constant boiling HCl for 24 hours. The hydrolyzate was filtered through a sintered glass funnel and evaporated to dryness under vacuum at 40°. The residue was made up to a desired volume and a portion of this was purified on the H^+ column in studies in-volving labeled compounds.

Chlorophylls: All the operations from extraction to separation and measurement of chlorophyll were performed in the dark or under a green safe light (Whitehouse and Zalik, 1967). The etiolated shoots after incubating in glycine-2-^{14}C and ALA-4-^{14}C under light were washed thoroughly with distilled water and ground finely in 80% acetone with a mortar and pestle. Acid washed sand was used as an abrasive and about 0.5 g of $CaCO_3$ was added to prevent pheophytin formation during maceration (Holden, 1965). The chlorophyll was further extracted with 80% acetone and made up to 25 ml. A 5 ml aliquot of this was evaporated to dryness and made up to 2 ml with a mixture of 1:1 petroleum ether and benzene (v/v). To separate chlorophyll from carotenoids and other pigments it was placed on a sucrose column and a 1:1 mixture of benzene and petroleum ether was forced through the column (Smith and Benitez, 1955). When all the carotenoids had been removed, the chlorophylls separated on the column were eluted with anhydrous acetone. An aliquot of this eluate was

read on the spectrophotometer (Beckman DK-1) and the presence of
the major chlorophyll peaks was confirmed.

V.   Separation of Chloroplast Protein

The chloroplasts were isolated from 6- and 10-day old seed-
lings grown in light. Leaves were macerated with a mortar and pestle
in a chilled phosphate buffer solution of pH 6.8 containing 0.33 M sucrose,
0.1 M Na_2HPO_4-KH_2PO_4, 0.1% NaCl and 0.1% $MgCl_2$ (Bucke et al., 1966).
The brei was filtered through 8-10 layers of cheese cloth. The filtrate
was transferred to 50 ml centrifuge tubes and centrifuged for 1
minute at 100 g at 4°. The supernatant was centrifuged for 15 minutes
at 1000 g. The pellet was suspended in buffer and centrifuged for
15 minutes at 1000 g to yield a crude chloroplast pellet. It was sus-
pended in a few ml of buffer and layered on a discontinuous sucrose
density gradient of 1.0, 1.5, 2.0 and 2.5 M in phosphate buffer. It
was centrifuged at 25,000 g for 20 minutes and individual layers were
examined under a light microscope (phase contrast) to determine the
layer of intact chloroplasts. The layer was removed with a syringe
and diluted with phosphate buffer not containing sucrose and centrifuged
at 3000 g for 10 minutes. The supernatant was discarded and the pellet
suspended in 0.33 M sucrose buffer and centrifuged at 1000 g for
15 minutes. The pellet was taken up in buffer and the protein pre-
cipitated with an equal volume of 10% trichloroacetic acid. The pre-
cipitated protein was washed 3 times with water and the protein
pellet used for determining amino acid composition.

VI. Measurements

1. Free amino acids

To determine the amounts of free amino acids in the embryo and endosperm of etiolated seedlings at various stages of development the lipid free soluble fraction was made up to 10 ml. An aliquot was diluted with an equal volume of sample dilution buffer pH 2.2 (see Appendix for composition of buffer). One ml of this was analyzed with a Beckman/Spinco model 120 amino acid analyzer (accurary ± 3%). The amino acids were eluted with citrate buffers of pH 3.25, 4.25 and 5.28 (see Appendix for composition of the buffers).

With this procedure the amides glutamine and asparagine were eluted together with serine and threonine. In order to obtain re-solution of these compounds a 1 ml portion of the lipid free soluble material was hydrolyzed for 3 hours with 1 ml of 2N HCl. This hydro-lysis resulted in the conversion of asparagine and glutamine into aspartic acid and glutamic acid respectively. The difference in the amounts of aspartic and glutamic acid before and after hydrolysis gave the amounts of asparagine and glutamine respectively (Miller, 1965).

In the case of studies involving labeled compounds the re-solution of free amino acids was obtained after hydrolysis of the purified amino acid fraction with 1N HCl for 3 hours.

2. Protein amino acids

The determination of protein amino acids of the endosperm and the embryo of etiolated seedlings was done on the hydrolyzate of the pellet. The hydrolyzate after drying was made up in distilled water, neutralized with KOH pellets and then diluted with sample dilution buffer pH 2.2 to a desirable volume. A portion of this was put on the

columns of the amino acid analyzer.

3. Total soluble sugars

The sugars were determined by the phenol sulfuric acid method

of Dubois et al. (1956). The purified fraction was diluted to a proper

concentration. To a 2 ml aliquot 1 ml of 5% phenol (w/v) was added.

After mixing, 5 ml of concentrated sulfuric acid were added with an

automatic pipette. The contents of the flask were shaken and kept at

room temperature for 10 minutes, with intermittent shaking. This was

followed by incubation at 30° for 30 minutes. The absorbance of the

solution was read in a Beckman DK-1 Spectrophotometer at 490 mμ. The

concentrations of sugars were calculated from a standard curve for

glucose (fig 3). In order to obtain consistent duplication of results

the addition of sulfuric acid had to be made quickly so as to develop

maximum heat.

4. Gas liquid chromatography of organic acids

The method as described by Canvin (1965) was employed with a

few modifications. The purified organic acid fraction obtained from

45 shoots or roots plus endosperm was evaporated to dryness under

vacuum at 40°. The residue was made up to 2 ml in methanol.

Esterification: One ml of the organic acid sample in methanol

was diluted with 9 ml of diethyl ether. This was esterified by

bubbling diazomethane freshly liberated from a mixture of 10 ml of

37.5% KOH plus 7 ml of 95% ethanol and 1 g of diazald (obtained from

Aldrich Chemical Co. Inc.) through it. The reaction was continued

until the yellow color in the sample persisted. The esterified
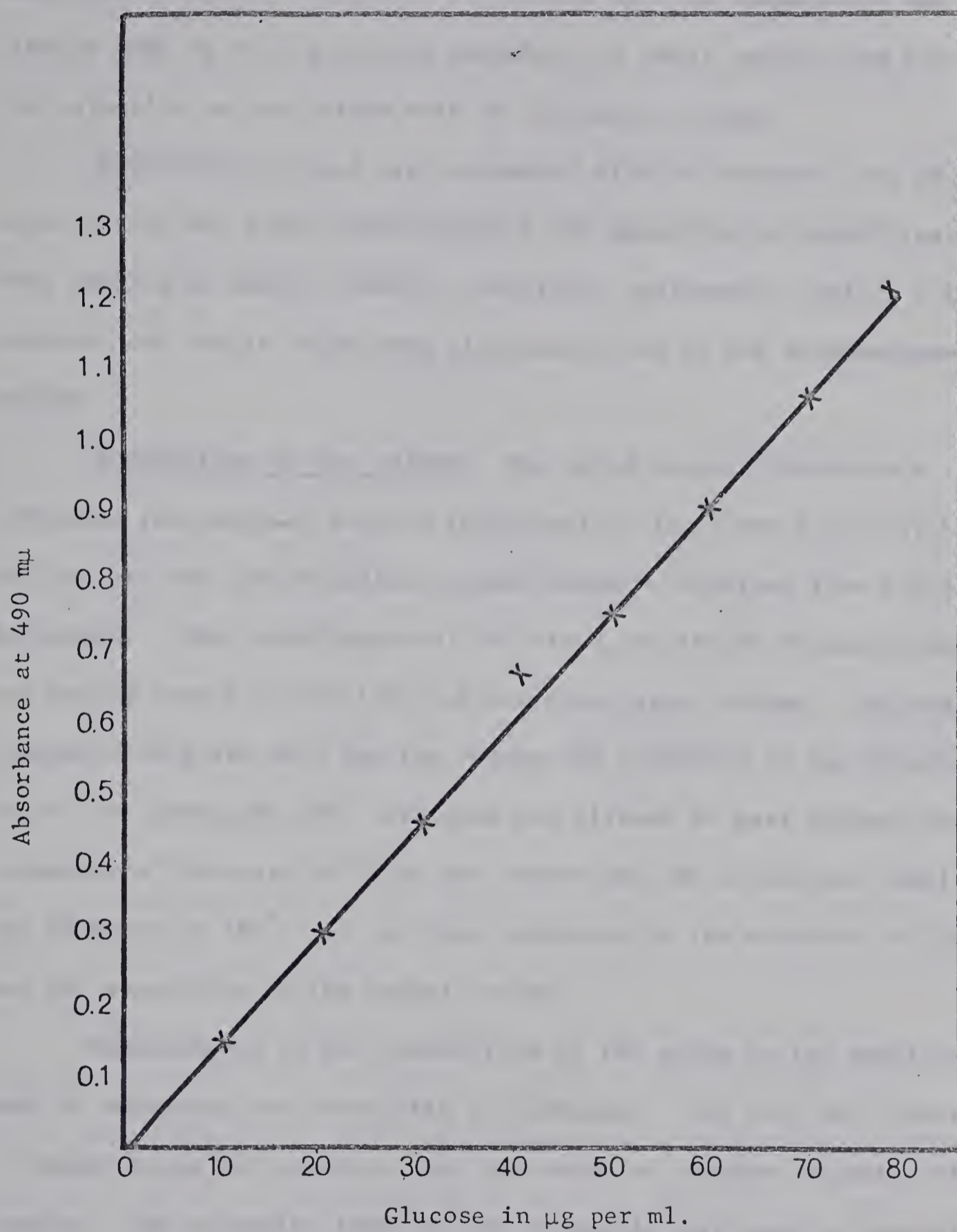
FIG.3. Standard curve for glucose.

sample was removed, evaporated to dryness at room temperature and finally made up to 0.5 ml with methanol. A small portion was used for injection on the column with an automatic syringe.

Esterification was also attempted with HCl-methanol and BF_3-methanol but was found unsatisfactory for quantitative esterification. Pure samples of oxalic, fumaric, succinic, oxaloacetic, malic, α-keto-glutaric and citric acids were also esterified by the diazomethane method.

Preparation of the column: The solid support Gas-chrom A (obtained from Applied Science Laboratories, Inc.) was coated with 10% Reoplex 400 (Polypropylene glycol adipate, obtained from Varian Aerograph). The coated material was sized and the 60-80 mesh fraction was packed into a 5 foot 1/8 inch stainless steel column. The ends were plugged with glass wool and the column was connected to the injector end of the Aerograph 200. Nitrogen was allowed to pass through the column at a flow rate of 25 ml per minute and the column was conditioned for 48 hours at 180°. It was then connected to the detector end and used for separation of the organic acids.

Measurements of the quantitites of the acids in the sample were made by measuring the areas with a planimeter. The area was converted to amount in mg by comparing with the areas of standard organic acid samples. The retention times of the standards were used to identify the peaks of the acids obtained from the plant material. Ten and 20 mg of each acid were esterified and different dilutions injected. Proportional dilutions gave identical responses for the amounts injected.

5. Chlorophyll

The chlorophyll measurements were done on 80% acetone extracts by the method of Arnon (1949) using the spectrophotometer.

6. Radioactivity

The plant material as described earlier was fractionated into the 6 fractions shown below.

```
                         Plant material
                                |
       _____|_____
      |                                                     |
  80% ethanol soluble                                   Insoluble
      |                                                     |
  ____|_____                 ___|_____
 |         |           |              |                 |           |
Ether    Organic     Sugars        Amino          Ether soluble   Pellet
soluble   acids                    acids                              |
                                                       (2)            |
 (1)       (4)         (3)          (5)                                |
                                                                 Hydrolyzate
                                                                      |
                                                       _____|_____
                                                      |                        |
                                                   Protein               Remainder
                                                   amino acids           disregarded
                                                      (6)
```
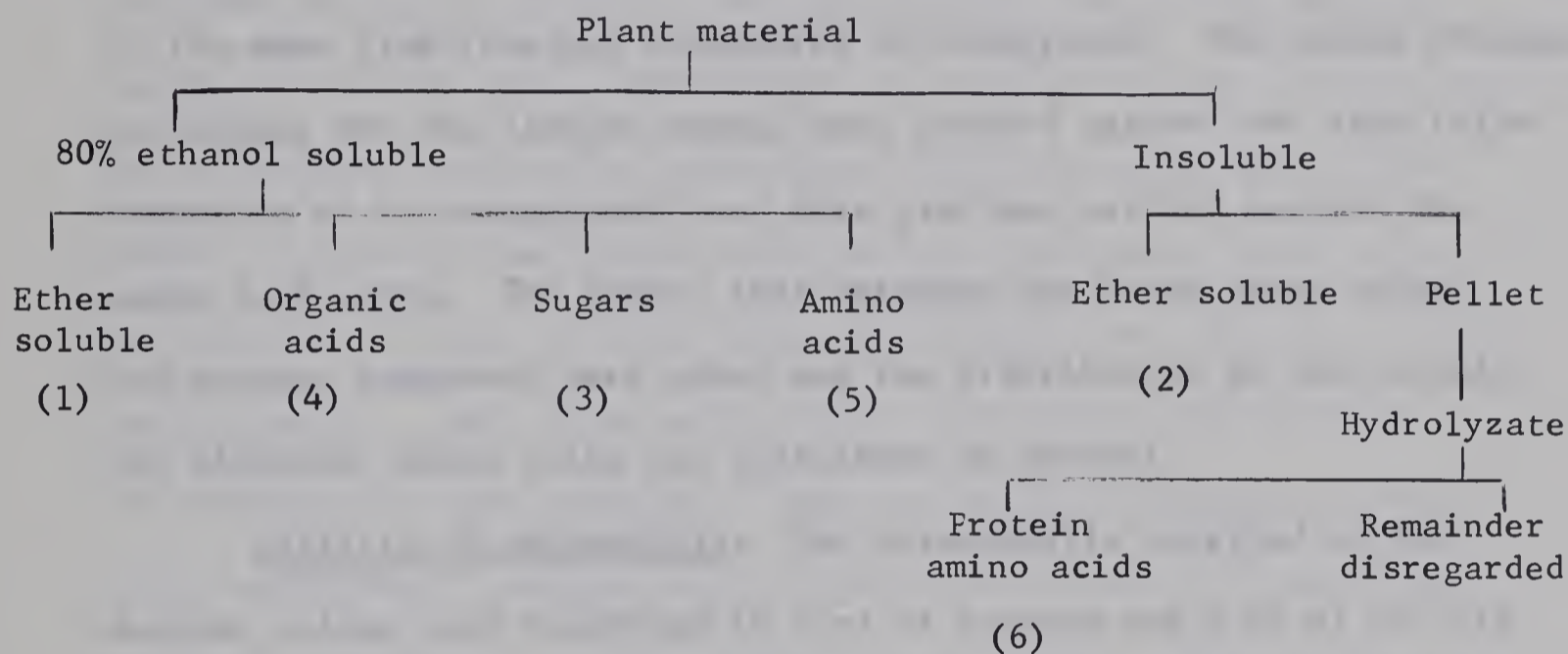
The activity for each fraction was obtained by measuring the activity of an aliquot of each. For this purpose the fractions were dried and made up to a desired volume. Samples of 0.05 ml were applied to planchets with a hypodermic syringe and the sample was dried at room temperature. The radioactivity was counted with a gas flow counter and no allowance was made for self absorption. Each sample was counted three times. The counts were corrected for background and the total counts incorporated in different fractions were calculated.

Measurement of activity of individual amino acids: The activity of individual amino acids was counted by passing the effluent from the

resin column of the amino acid analyzer through a scintillation flow cell counter (Nuclear Chicago). The counts on the effluent were recorded every minute. The effluent was then directed through the reaction bath of the amino acid analyzer for development of color and the quantities of individual amino acids were obtained in the usual manner. A count obtained by passing through just the buffer at the same flow rate was considered as background. The counts obtained per minute for the labeled sample were plotted against the time (after deduction of the background) and this plot was matched against the amino acid curve. The counts thus obtained for known amino acids and unknown compounds were added and the distribution of the activity for different amino acids was calculated as percent.

Activity of chlorophyll: The chlorophylls purified on the sucrose column were dissolved in 1 ml of acetone and 0.05 ml of this was applied to a planchet and the activity was counted with a gas flow counter.

RESULTS AND DISCUSSION

I. Free and Protein Amino Acids During Germination

As the seeds were germinated in the dark and in the absence

of any external supply of nitrogen the results of amino acid

analyses are being expressed as micromoles of amino acid per gram

weight of the dry seed. The values are averages of two replicates,

the difference between the replicates being less than 10% in most

cases. It was generally observed that the difference between replicates

was more in the case of those amino acids which were present in very

small amounts. Only those amino acids that could be identified

accurately have been reported. Quantitative determination of trypto-

phan, methionine, cysteine and cystine were erratic and hence they

were not included. The 80% ethanol soluble amino acids have been

considered as free amino acids and the remainder as protein amino

acids.

(a) Changes in the endosperm

Protein amino acids: It may be seen that all the protein amino

acids decreased as germination progressed so that by the end of the

10th day very little was left as protein (table I, figs. 4 and 5).

The maximum decrease in net amount of amino acids occurred between

the 2nd and 4th day. Quantitatively glutamic acid and proline

appeared to be very important as amino acids of the reserve protein.

From the point of view of nitrogen glutamic acid was the most

important followed by arginine. In general the endosperm of the

Table I. Protein amino acids of the endosperm of etiolated barley
          seedlings at various stages of development during germination.

Days Amino Acid		0	2	4	6	8	10
		μmoles/g seed					
Lysine	N	37.1	25.8	17.4	7.6	4.2	1.4
	M	47.2	25.5	16.7	9.3	3.1	1.9
Histidine	N	17.0	18.4	9.1	3.7	1.7	0.7
	M	17.2	18.2	8.2	5.3	1.2	0.7
Arginine	N	45.0	31.0	18.2	7.7	4.1	1.2
	M	33.9	28.8	16.9	9.2	3.0	1.6
Aspartic	N	59.4	48.1	27.5	15.1	7.9	3.4
	M	59.1	43.7	29.5	17.2	7.5	4.4
Threonine	N	44.6	35.1	16.0	7.6	3.6	1.8
	M	37.7	33.9	16.8	8.5	3.5	2.1
Serine	N	61.8	53.7	21.7	9.6	4.6	2.2
	M	53.5	51.4	23.6	11.2	3.9	2.8
Glutamic	N	222.6	197.8	66.2	18.3	6.8	2.6
	M	162.6	170.7	68.3	21.2	8.4	4.1
Proline	N	119.4	109.9	34.3	10.1	4.2	2.0
	M	87.5	95.5	35.9	12.0	4.0	2.5
Glycine	N	82.9	64.3	39.3	14.6	7.6	3.7
	M	73.3	61.8	30.7	16.7	7.3	4.6
Alanine	N	64.6	52.6	25.8	12.6	6.3	3.1
	M	55.1	47.4	28.0	14.9	6.1	3.8
Valine	N	64.9	55.7	23.2	10.5	5.0	2.0
	M	51.1	50.0	25.2	15.8	4.9	2.8
Isoleucine	N	38.4	30.4	12.3	6.4	2.9	1.4
	M	28.4	28.6	14.8	7.3	3.0	1.8
Leucine	N	77.1	68.7	27.2	12.1	5.3	2.5
	M	60.0	60.0	30.0	14.1	5.4	3.2
Tyrosine	N	28.8	23.9	9.4	4.2	2.0	0.8
	M	22.8	20.2	9.4	5.0	2.1	1.0
Phenylalanine	N	43.9	40.0	16.0	6.5	2.8	1.3
	M	32.7	32.5	15.7	7.6	2.9	1.7
TOTAL	N	1007.5	855.4	363.6	146.6	69.0	30.1
	M	822.1	768.2	369.7	175.3	66.3	39.0

N = Normal, M = Mutant
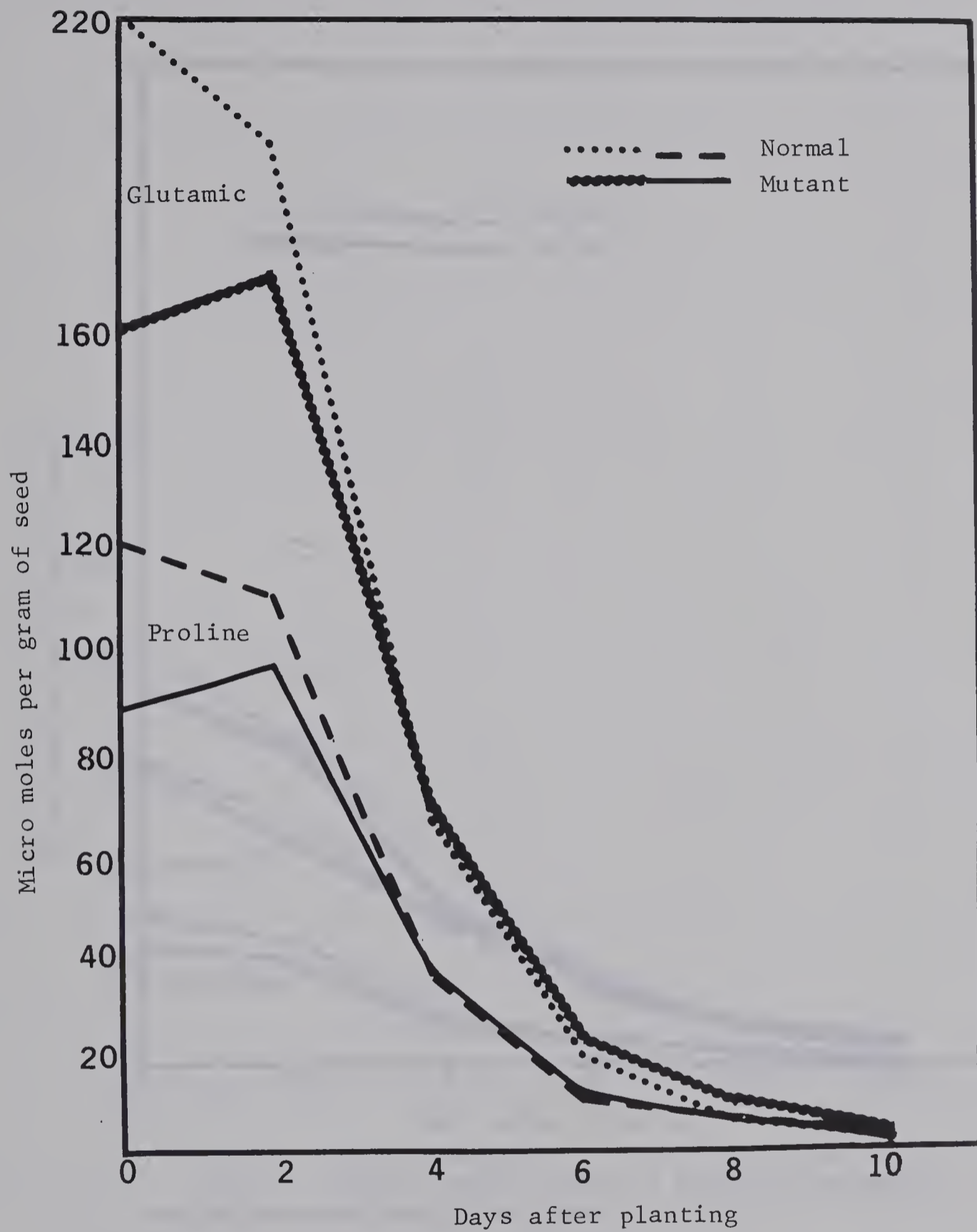
FIG. 4. Protein amino acids of barley endosperms during germination in the dark.
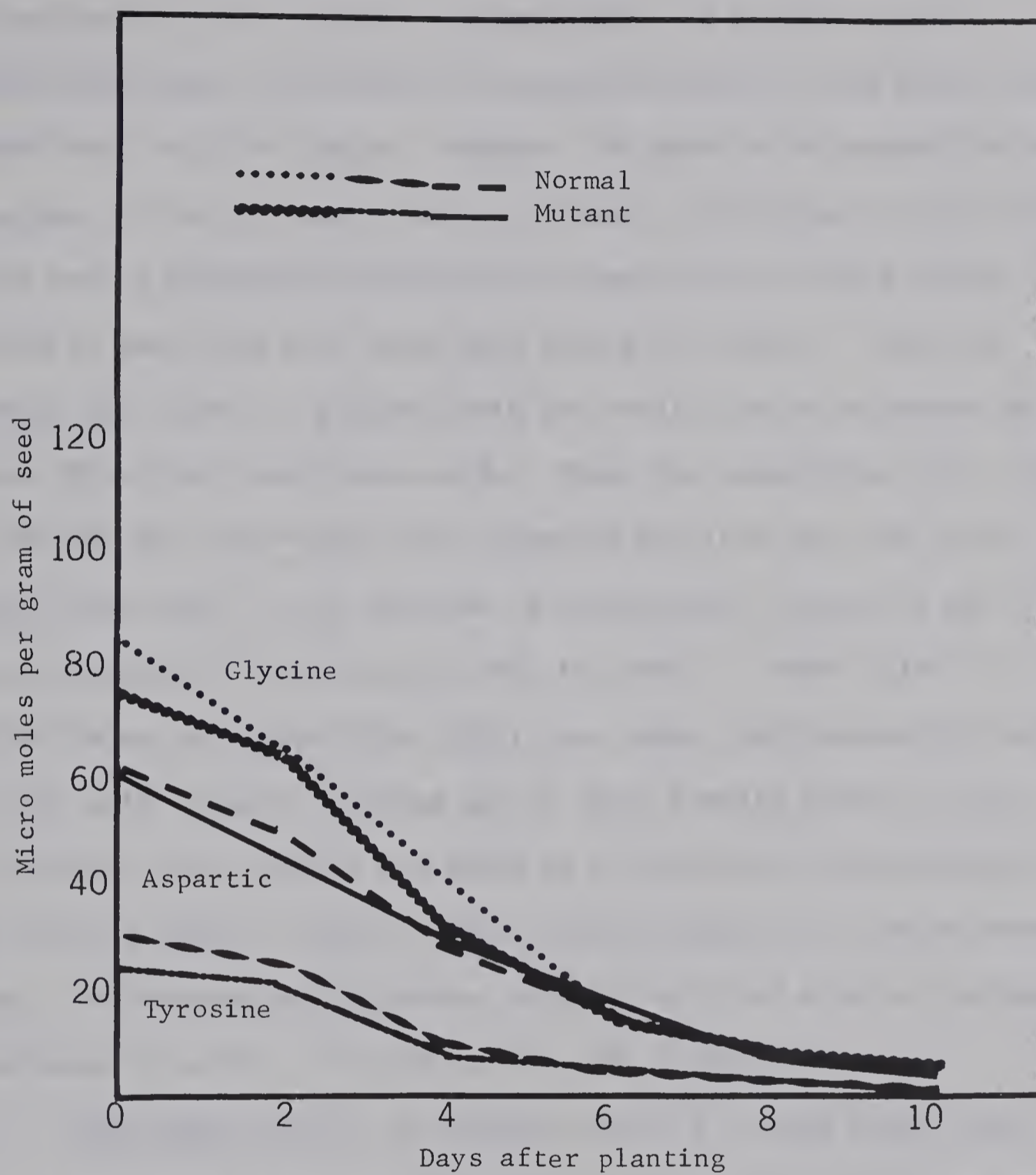
FIG.5. Protein amino acids of barley endosperms during germination in the dark.

ungerminated normal seed was richer in protein amino acids than
the endosperm of the mutant. Glutamic acid and proline together
constituted about 342 μmoles in the normal whereas in the mutant they
constituted only 250 μmoles. However, the amino acid composition as
a percent of the protein at various stages of development showed that
there were no significant differences between the two lines (table II).
It may be seen from this table that during the first 2 days the
protein was richer in glutamic acid and proline which accounted for
about 30% of the total amino acids. These two constituted about 28%
on the 4th day after which they accounted for less than 20% of the
total amino acids. This decrease is considerable and may be due to
the utilization of some protein rich in these 2 amino acids. In
barley Folkes and Yemm (1956, 1958) have shown that hordein and hordenin
are the major reserve proteins and of these hordein which is richer
in glutamic acid, proline and amide is utilized first during germination.
The results shown in table II lend indirect support to such an observa-
tion. The percentage of glycine, aspartic acid and alanine increased
from about 8 to 12%, 7 to 11% and 7 to 10% respectively.

Free amino acids: The changes observed in free amino acids
of the endosperm of etiolated seedlings of the normal and the mutant
at various stages of development are given in table III and figs 6 and 7.
The pattern of behaviour of any amino acid as well as the actual amount
present in either the normal or the mutant was the same. Quantitative-
ly the amide glutamine and the amino acid proline appeared to be most
important. In ungerminated seed the amount of free proline was
particularly notable. Most other amino acids excepting aspartic,

Table II. Amino acid composition of reserve protein of the endosperm
of etiolated barley seedlings at various stages of
development during germination.

Days Amino Acid		Mole % amino acid					
		0	2	4	6	8	10
Lysine	N	3.7	3.0	4.8	5.2	6.1	4.8
	M	5.7	3.3	4.5	5.3	4.6	4.8
Histidine	N	1.7	2.2	2.5	2.5	2.5	2.4
	M	2.1	2.4	2.2	3.0	1.9	1.9
Arginine	N	4.5	3.6	5.0	5.3	5.9	4.0
	M	4.1	3.8	4.6	5.2	4.5	4.2
Aspartic	N	5.9	5.6	7.5	10.3	11.5	11.1
	M	7.2	5.7	8.0	9.8	11.3	11.3
Threonine	N	4.4	4.1	4.4	5.2	5.2	5.8
	M	4.6	4.4	4.5	4.9	5.3	5.4
Serine	N	6.1	6.3	6.0	6.5	6.7	7.3
	M	6.5	6.7	6.4	6.4	5.8	7.1
Glutamic	N	22.1	23.1	18.2	12.5	9.8	8.7
	M	19.8	22.2	18.5	12.1	12.7	10.5
Proline	N	11.9	12.8	9.4	6.9	6.0	6.6
	M	10.6	12.4	9.7	6.8	6.0	6.4
Glycine	N	8.2	7.5	10.8	10.0	11.0	12.3
	M	8.9	8.1	8.3	9.5	11.1	11.7
Alanine	N	6.4	6.2	7.1	8.6	9.2	10.1
	M	6.7	6.2	7.6	8.5	9.2	9.8
Valine	N	6.4	6.5	6.4	7.2	7.2	6.7
	M	6.2	6.5	6.8	9.0	7.4	7.2
Isoleucine	N	3.8	3.6	3.4	4.4	4.2	4.7
	M	3.5	3.7	4.0	4.2	4.5	4.6
Leucine	N	7.7	8.0	7.5	8.2	7.7	8.4
	M	7.3	7.8	8.1	8.0	8.2	8.1
Tyrosine	N	2.8	2.8	2.6	2.8	2.9	2.7
	M	2.8	2.6	2.5	2.9	3.2	2.6
Phenylalanine	N	4.4	4.7	4.4	4.4	4.1	4.4
	M	4.0	4.2	4.3	4.4	4.3	4.4

N = Normal, M = Mutant

Table III. Free amino acids of the endosperm of etiolated barley seedlings at various stages of development during germination.

| Days Amino Acid		μmoles/g seed					
		0	2	4	6	8	10
Lysine	N	0.3 =	1.0	1.3	1.0	0.7	T
	M	0.3	0.9	1.6	0.9	0.8	0.2
Histidine	N	0.2	0.7	0.8	0.4	0.2	T
	M	0.3	0.7	0.8	0.3	0.3	0.1
Arginine	N	0.8	1.2	1.4	1.0	0.4	T
	M	0.8	1.3	1.3	1.0	0.4	0.2
Aspartic	N	1.8	1.7	1.4	1.1	0.7	0.2
	M	2.1	1.9	1.6	1.2	0.9	0.4
Threonine	N	0.2	1.3	1.6	1.0	0.6	0.1
	M	0.2	1.3	1.5	1.0	0.7	0.3
Serine	N	0.7	2.0	2.0	1.3	0.9	0.3
	M	0.5	1.9	2.4	1.4	0.9	0.7
Glutamic	N	0.8	2.6	1.2	0.8	0.5	0.2
	M	0.9	2.9	1.2	0.9	0.6	0.3
Proline	N	4.5	6.8	7.7	2.4	0.5	0.1
	M	5.5	7.8	7.5	3.2	0.6	0.1
Glycine	N	0.3	0.9	0.8	0.6	0.4	T
	M	0.3	0.9	0.8	0.6	0.5	0.2
Alanine	N	0.8	2.9	2.0	1.4	0.6	0.2
	M	1.0	3.5	2.1	1.5	1.0	0.3
Valine	N	0.8	2.2	3.2	1.3	0.7	0.2
	M	0.8	2.4	3.1	1.4	0.9	0.3
Isoleucine	N	0.2	1.5	2.2	1.0	0.4	0.1
	M	0.3	1.5	2.5	1.1	0.5	0.2
Leucine	N	0.3	3.2	4.3	1.8	0.9	0.1
	M	0.4	3.2	4.3	2.0	1.0	0.3
Tyrosine	N	0.1	1.8	3.4	1.1	0.4	0.1
	M	0.1	1.8	3.2	1.2	0.5	0.2
Phenylalanine	N	0.3	2.6	4.6	1.7	0.6	0.1
	M	0.3	2.6	4.3	1.9	0.6	0.2
Asparagine	N	2.4	3.4	2.8	1.9	2.0	0.9
	M	1.1	3.2	2.3	1.7	1.9	1.4
Glutamine	N	2.5	8.4	8.6	2.2	1.1	0.4
	M	1.8	7.5	9.3	2.9	0.9	1.2

N = Normal, M = Mutant, T = Trace
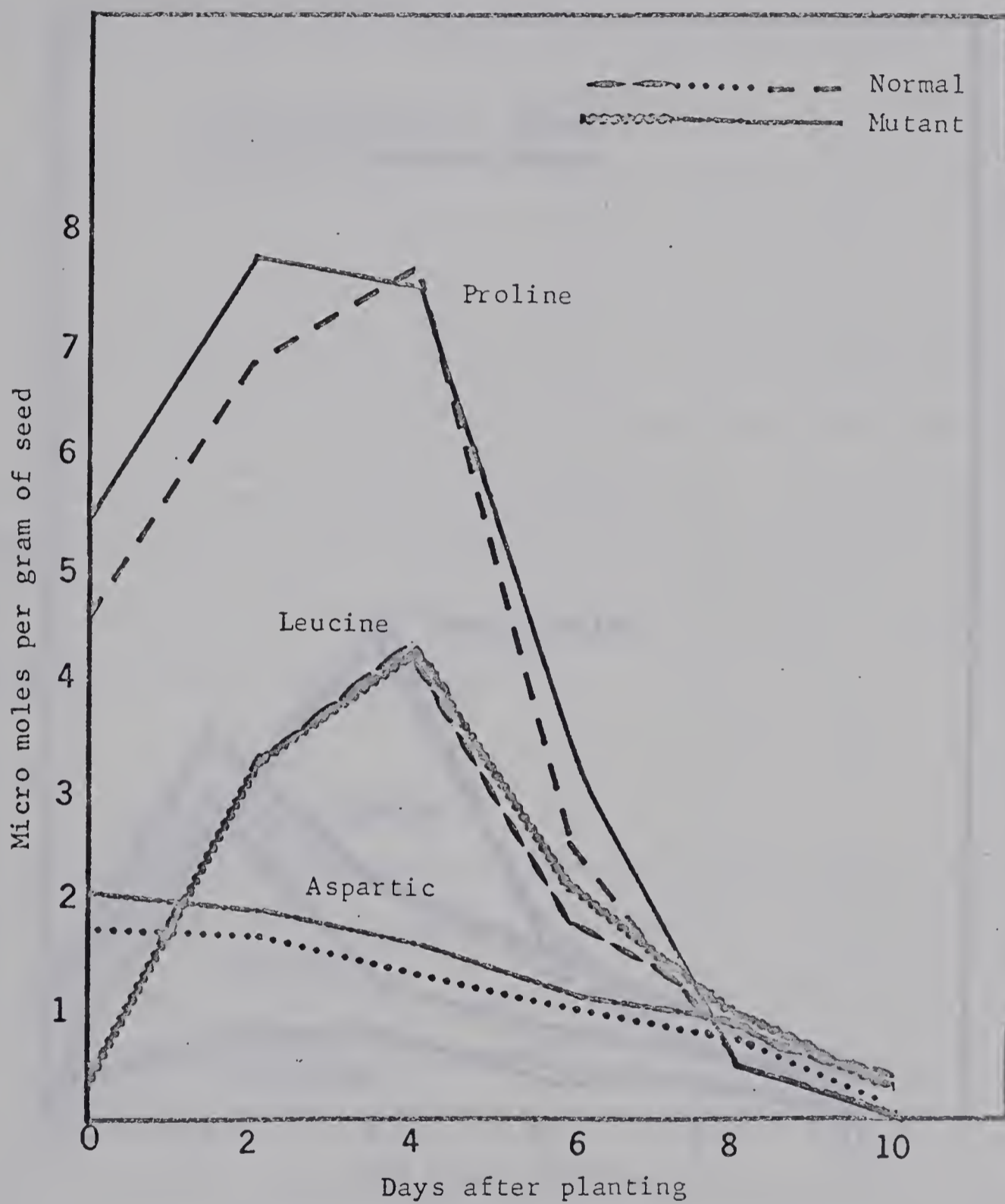
FIG. 6. Free amino acids of barley endosperms during germination in the dark.
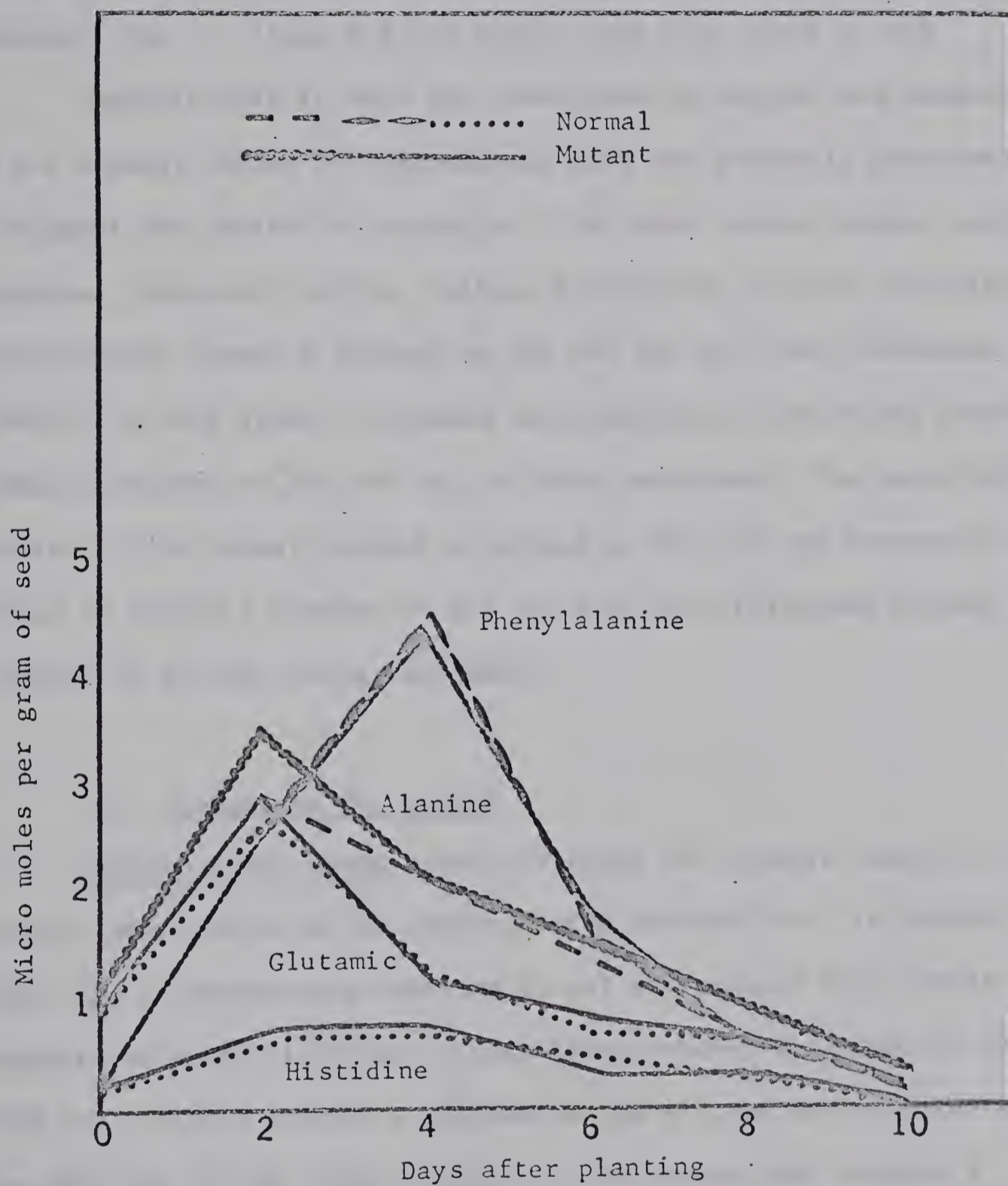
FIG. 7. Free amino acids of barley endosperms during germination in the dark.

asparagine and glutamine were present in very low amounts. At the end of 10 days all the amino acids were present in extremely small amounts. The 2 lines did not differ from each other at all.

Aspartic acid in both the normal and the mutant was present in the highest amount in ungerminated seed and gradually decreased throughout the period of etiolation. The amino acids lysine, histidine, arginine, threonine, serine, valine, isoleucine, leucine, tyrosine, phenylalanine showed a maximum on the 4th day and then diminished in quantity in both lines. Glutamic acid, glycine, alanine and asparagine showed a maximum on the 4th day and then decreased. The amino acid proline in the normal reached a maximum on the 4th day whereas in the mutant it showed a maximum on the 2nd day; the difference between the 2nd and the 4th day being very small.

(b) Changes in the embryo

Protein amino acids: Table IV shows the changes observed in protein amino acids of the embryo during germination. In general there was a considerable increase in all the protein amino acids. Aspartic acid and histidine in both lines reached a maximum on the 10th day. Lysine reached a maximum on the 4th day and arginine on the 10th day in the normal whereas in the mutant both reached a maximum on the 6th day. The quantities of these amino acids from the 6th day onwards did not change much. All the other amino acids showed a maximum on the 6th day after which there was a slight decline.

There was little difference between the amounts of individual

Table IV. Protein amino acids of the embryo of etiolated barley
seedlings at various stages of development during
germination.

Days Amino Acid		μmoles/g seed					
		0	2	4	6	8	10
Lysine	N	4.8	11.1	38.0	32.5	32.5	32.8
	M	4.6	11.2	29.9	37.2	30.9	33.5
Histidine	N	1.9	2.5	8.0	8.6	8.3	9.7
	M	1.6	3.5	7.0	8.1	8.2	8.6
Arginine	N	5.2	9.2	22.9	23.6	24.3	24.9
	M	5.1	8.6	21.3	27.6	22.3	25.1
Aspartic	N	6.7	14.3	40.2	52.4	47.2	65.1
	M	6.3	13.9	37.8	54.7	53.8	59.2
Threonine	N	4.0	8.3	22.8	29.1	25.3	26.0
	M	3.8	7.9	21.1	28.5	24.8	27.3
Serine	N	4.7	9.6	27.5	33.3	30.2	30.1
	M	4.8	9.1	24.7	34.1	29.5	30.8
Glutamic	N	9.4	15.9	42.6	52.0	44.6	44.0
	M	8.9	15.1	38.5	53.4	44.0	42.6
Proline	N	3.2	7.9	21.6	23.7	22.6	22.5
	M	3.0	8.1	19.3	28.8	22.3	22.7
Glycine	N	8.8	15.5	41.9	52.1	47.5	42.7
	M	8.4	15.0	39.2	52.5	46.9	47.5
Alanine	N	7.4	15.2	42.3	53.1	47.9	48.0
	M	7.5	14.0	39.7	54.0	47.2	48.2
Valine	N	5.0	10.9	30.4	36.9	34.3	33.3
	M	4.7	10.4	27.0	38.0	33.4	31.6
Isoleucine	N	2.6	6.2	17.8	23.7	21.8	21.3
	M	2.7	6.3	17.4	22.1	21.0	20.4
Leucine	N	5.3	12.2	34.6	44.2	40.2	37.6
	M	5.4	12.0	32.8	43.3	40.0	36.6
Tyrosine	N	1.7	3.8	11.4	13.2	12.6	10.6
	M	1.6	3.4	10.2	13.7	11.9	10.6
Phenylalanine	N	2.6	5.9	16.6	20.3	19.1	18.0
	M	2.7	5.4	15.0	20.9	18.3	18.2

N = Normal, M = Mutant

protein amino acid present on any day between the normal and the mutant showing that the pattern of metabolism of amino acids and protein between the two lines when grown in the dark was not different.

Free amino acids: During germination all of the amino acids showed an increase (table V, figs 8 and 9). The free amino acid content of the embryo prior to germination was extremely low. From the point of view of nitrogen, arginine and asparagine followed by proline were the most important amino acids of the seed embryo. The amino acids lysine, glycine, isoleucine, leucine and phenylalanine showed a maximum on the 8th day whereas arginine, aspartic acid and asparagine showed a maximum on the 10th day in both lines. For both lines the amount of arginine decreased to a trace on the 8th day and increased suddenly again on the 10th day. Glutamine showed a maximum on the 6th day and then declined. Proline gradually increased reaching a maximum on the 4th day after which it declined and was present in least amount of all the amino acids on the 10th day.

The amino acids threonine and serine showed a maximum on day 8 in the normal and on day 10 in the mutant whereas valine and tyrosine in the normal reached a maximum on the 10th day and in the mutant on the 8th day. Alanine reached a maximum on the 6th day in the normal but on the 8th day in the mutant.

The amide asparagine accumulated throughout the period of germination and represented about 50% of the total free amino acids on the 10th day. The loss of aspartic acid from the reserve proteins of the endosperm (table I) was far less than the increase observed in asparagine, therefore, all the asparagine could not have arisen

Table V. Free amino acids of the embryo of etiolated barley seedlings at various stages of development during germination.

Days Amino Acid		μmoles/g seed					
		0	2	4	6	8	10
Lysine	N	0.1	0.4	1.5	4.0	8.8	5.4
	M	0.1	0.4	1.4	2.7	8.7	5.5
Histidine	N	T	0.4	2.6	7.6	9.8	10.3
	M	0.1	0.5	2.7	5.8	13.1	11.1
Arginine	N	0.7	1.4	0.9	0.7	0.2	4.2
	M	0.6	1.3	1.0	1.4	T	2.9
Aspartic	N	T	0.4	2.9	3.5	4.8	13.1
	M	T	0.4	3.5	4.4	5.5	15.2
Threonine	N	0.1	1.0	2.7	11.9	24.7	16.3
	M	0.1	0.9	4.3	8.9	14.3	16.0
Serine	N	0.2	1.4	3.0	14.2	28.4	25.7
	M	0.2	1.1	4.7	13.4	22.0	22.8
Glutamic	N	0.5	2.6	5.7	6.3	5.3	6.1
	M	0.6	2.6	6.2	6.7	5.5	5.8
Proline	N	1.1	2.2	4.0	2.4	1.9	0.6
	M	1.2	2.1	4.0	2.8	2.0	0.7
Glycine	N	0.1	0.5	2.0	4.3	6.1	3.6
	M	0.1	0.5	1.8	4.1	5.8	3.7
Alanine	N	0.3	4.0	7.4	19.3	18.4	11.6
	M	0.4	5.0	6.9	18.0	21.5	11.6
Valine	N	0.1	2.0	10.2	16.0	21.7	22.1
	M	0.1	1.9	9.8	16.1	25.8	20.8
Isoleucine	N	-	1.3	6.6	9.6	10.9	9.5
	M	-	1.1	6.3	9.3	10.9	9.3
Leucine	N	-	2.8	8.8	9.1	10.8	4.9
	M	-	2.0	8.2	9.1	10.9	5.2
Tyrosine	N	-	0.5	2.4	3.5	5.0	5.3
	M	-	0.5	2.3	3.6	5.0	4.8
Phenylalanine	N	-	0.7	2.5	3.4	4.8	4.7
	M	-	0.6	2.5	3.6	5.0	4.2
Asparagine	N	1.3	3.2	21.5	94.3	155.9	185.8
	M	1.2	2.6	21.4	90.0	135.3	166.9
Glutamine	N	0.2	7.4	19.2	39.7	30.8	18.1
	M	0.2	5.7	17.0	39.4	23.4	20.8

N = Normal, M = Mutant, T = Trace
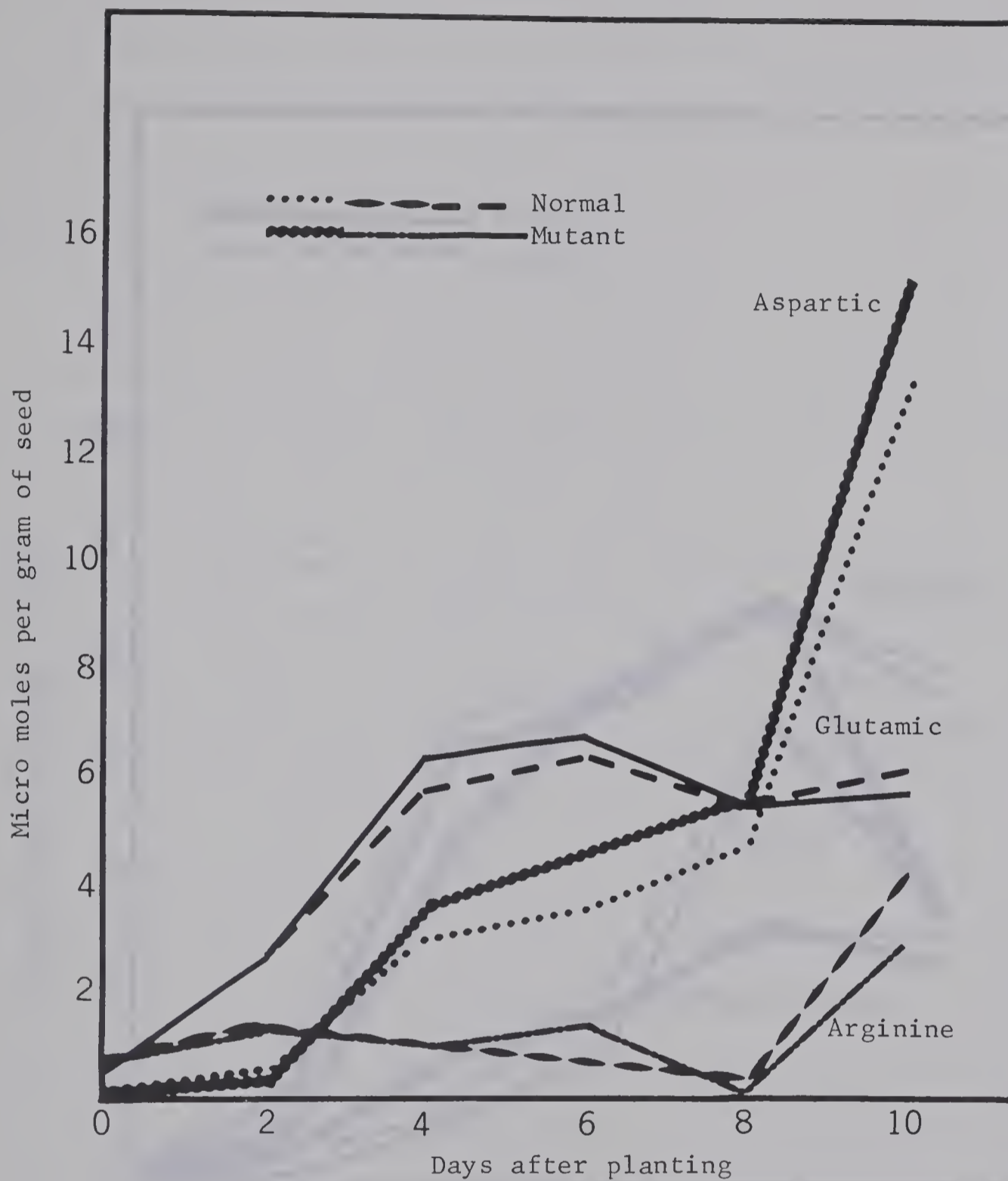
FIG. 8. Free amino acids of barley embryos during germination in the dark.
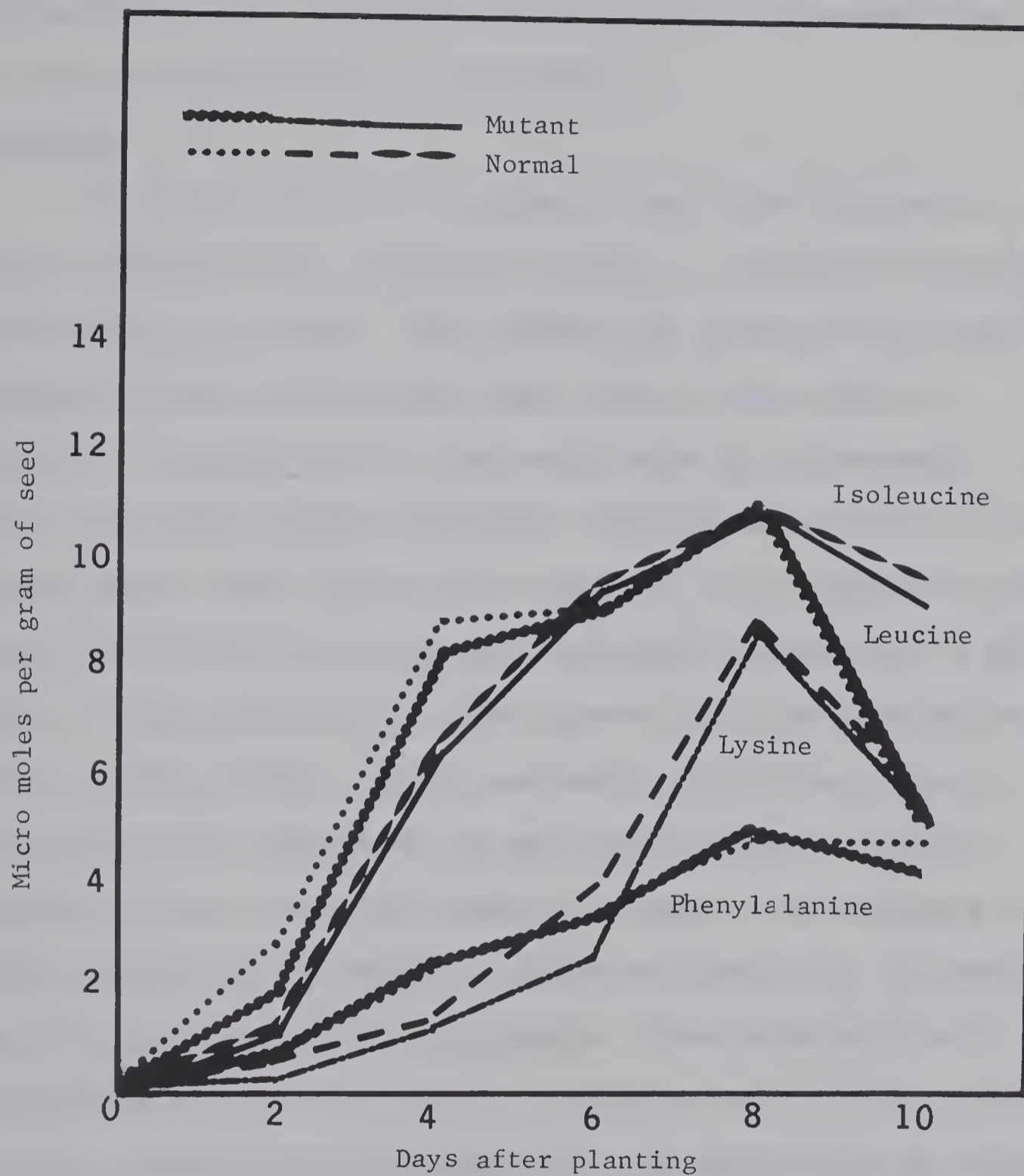
FIG. 9. Free amino acids of barley embryos
during germination in the dark.

directly from aspartic acid. The free amino acid content of

etiolated embryos of the two lines did not differ much on any day.

From tables IV and V it may be observed that in the mutant

glycine which is the nitrogenous building block of chlorophyll was

not deficient when compared to the normal.

Discussion

The results presented in tables IV and V show that over a

10-day period there was a many fold increase in the free and protein

amino acids of the embryo. This increase was accompanied by a con-

comitant decrease in the protein amino acids of the endosperm

(table I). A gradual rise in free amino acids of the endosperm

until 4 days and a decrease thereafter could not account for all the

loss of protein amino acids of the endosperm. This suggests that the

protein hydrolyzed in the endosperm was rapidly translocated to the

embryo. These results are in close agreement with the observations of

Folkes and Yemm (1958). Stewart and Beevers (1967) have recently

shown that in the castor bean the amino acids glutamate, glycine,

aspartate, alanine and serine underwent changes in the endosperm to

produce sucrose and the ammonia released on deamination, and some of

the carbon was translocated as glutamine. Other amino acids were

translocated to the embryo intact. Asparagine was not found to function

as an NH_2 carrier whereas glutamine was. The maximum loss of protein

amino acids of the endosperm occurred between 2 and 4 days and during

this period the maximum increase in protein amino acids of the embryo

took place. The amino acid composition of the reserve protein (table I,

0 day) and embryonic protein (table IV, 10 day) was very different.

This indicates that prior to incorporation of amino acids into the

embryo some interconversion must have taken place. Similar conclusions

were drawn by Folkes and Yemm (1958) from their results.

From the data given in tables I, III, IV and V it is possible
to draw balance sheets for each individual amino acid by adding the
value for the amino acid under consideration from all these tables.
The results of such calculations are presented in table VI. The de-
crease or increase in each amino acid during the 10-day period has
been shown in column 3. It is seen that the only amino acid showing
a great increase was aspartic acid (including asparagine). The other
amino acids showed losses. The most striking decreases occurred in
glutamic acid and proline followed by glycine and leucine. Maximum
losses in glutamic acid and proline were also noticed by Folkes and
Yemm (1958) in light grown seedlings. However, they observed in-
creases in most other amino acids. The only amino acids that showed
decreases in their work were cystine, glutamic acid, leucine, phenyl-
alanine, serine and proline. Under the conditions of this experiment
the growth of the seedling was entirely supported by the reserves in
the endosperm. Consequently at later stages carbon from amino acids
was probably utilized for respiration and synthesis of other compounds.
Under such conditions nitrogen liberated from amino acids would be
bound in a non-toxic form as asparagine. The continuous increase in
asparagine throughout the 10-day period supports this. As compared
to asparagine accumulation glutamine accumulation in the free form
was negligible. Several workers have shown that in etiolated plants
asparagine accumulation is favoured (Meiss, 1952). However, if there
is an abundant supply of sugars glutamine synthesis takes place
(Yemm, 1949). Decrease in the accumulation of glutamine after 4 days
may be taken as further evidence that the amino acids were being
used for respiration.

Table VI. Changes in amino acids of etiolated barley seedlings during germination.

| Days
Amino Acid | | μmoles/g seed | | |
		0	10	Net Change
Lysine	N	42.3	39.6	- 2.7
	M	52.2	41.1	-11.1
Histidine	N	19.1	20.7	+ 1.6
	M	19.2	20.5	+ 1.3
Arginine	N	51.7	30.3	-21.4
	M	40.4	29.8	-10.6
Aspartic + Asparagine	N	71.6	268.5	+196.9
	M	69.8	247.5	+177.7
Threonine	N	48.9	44.2	- 4.7
	M	41.8	45.7	+ 3.9
Serine	N	67.4	58.3	- 9.1
	M	59.0	57.1	- 1.9
Glutamic + Glutamine	N	236.0	71.4	-164.6
	M	175.0	74.8	-100.2
Proline	N	128.2	25.2	-103.0
	M	97.2	26.0	- 71.2
Glycine	N	92.1	50.0	-42.1
	M	82.1	56.0	-26.1
Alanine	N	73.1	62.9	-10.2
	M	64.0	63.9	- 0.1
Valine	N	70.8	57.6	-13.2
	M	56.7	55.5	- 1.2
Isoleucine	N	41.2	32.3	-8.9
	M	31.4	31.7	+0.3
Leucine	N	82.7	45.1	-37.6
	M	65.8	45.3	-20.5
Tyrosine	N	30.6	16.8	-13.8
	M	24.5	16.6	- 7.9
Phenylalanine	N	46.8	24.1	-22.7
	M	35.7	24.3	-11.4
TOTAL	N	1102.5	847.0	-255.5
	M	914.8	835.8	- 79.0

N = Normal, M = Mutant

During the 10-day period there was a loss of 255 μmoles of amino acids in the normal. Much of this loss of amino acids may be accounted for by the synthesis of other nitrogenous compounds like nucleic acids and porphyrins. The loss of glutamic acid and proline during the 10-day period deserves special attention. In Montcalm barley similar decreases in glutamic acid and proline were observed (Sane and Zalik, unpublished data). Glutamic acid has been shown to occupy a key position in the metabolism of amino acids. A large number of amino acids under suitable conditions can undergo trans-amination with α-ketoglutaric acid to form glutamic acid. The reaction is reversible and has been shown to occur in plants. Evidence in this respect has been reviewed by Kretovich (1958) and Fowden (1967). Thus glutamic acid may have undergone transamination reaction to give rise to other amino acids and the resultant α-ketoglutaric acid may have entered the TCA cycle. Glutamic acid can also undergo several other reactions (Meister, 1965) and be utilized for the synthesis of other compounds. Splittstoesser (1967) using maize tissues noticed that in the axis in 24 hours 89% of the glutamate absorbed was metabolized to CO_2, in the scutellum the corresponding figure was 76% and in the endosperm 37%.

Proline was not only important quantitatively in the reserve protein but it was also important as a free amino acid in the endosperm. On account of the importance of proline in barley reserve protein, its metabolism in Montcalm barley during germination was investigated (Sane and Zalik, unpublished data). It was noticed that proline injected into the endosperm of 3-day old etiolated seedlings was trans-

located into the embryo and some was transformed into glutamic acid.
Of the total label retained about 40% had been translocated to the
embryo in 3 hours. Of the total label present in the embryo about
65% was accounted for by protein and free amino acids and about 35%
was present in organic acids. In the endosperm organic acids con-
stituted about 12% to 14% of the total label. Thus proline from the
endosperm could be translocated to the embryo and be utilized for
protein synthesis and organic acid formation.

These studies were not conducted under aseptic conditions but
the observed metabolism probably actually reflects the usual metabolism
of such seedlings. Under strictly aseptic conditions the results may
have been different.

In view of the lower percent of proline in the embryonic protein
it is interesting to know why proline is present in such a high pro-
portion in the reserve protein. Sacktor and Childress (1967) have
shown that isolated mitochondria from flight muscles of insects are
permeable to proline, α-glycerophosphate and pyruvate but not to
glutamate and substrates of the TCA cycle. The results suggested
that proline enhanced the rate of pyruvate metabolism by penetrating
the mitochondrial barrier and forming the intramitochondrial pre-
cursors of oxaloacetate. Britikov et al. (1965) studying the trans-
formation of proline in germinating pollen and pistil tissues
characterized proline as a storage substance of manifold and complete
utilization. Proline may play a similar role in germinating seeds.

Aspartic acid and asparagine together increased more than
3 fold during the 10-day period. Aspartic acid may have originated

from glutamate by transamination. Yemm (1954, reported by Folkes and Yemm, 1958) has shown that the barley embryo is rich in trans-aminases. Leonard and Burris (1947) also showed that the extracts from developing barley embryos catalyzed reversible transamination between glutamate and oxaloacetate. Some asparagine may have come from asparagine of reserve protein and the additional may have been synthesized from aspartic acid by asparagine synthetase. This enzyme has been shown to occur in plants (Webster and Varner, 1955).

The translocation of amino acids from the endosperm to the embryo and their incorporation into proteins of embryo needs to be examined from the point of view of two theories forwarded for the synthesis of proteins in plants. The results obtained by Steward et al. (1956, 1958) led them to conclude that amino acids were not the direct precursors of protein. They had observed (1956) that the specific activities of protein glutamate, alanine and aspartate were higher when ^{14}C glucose was supplied to tissue cultures of carrot roots as compared to ^{14}C glutamine. They explained these results by suggesting that protein was synthesized mainly from carbon skeletons derived from glucose which combined with nitrogen groups and were immediately converted to protein at a site remote from the soluble amino acids. Webster (1959) disagreed with this explanation and suggested that such results could be due to the difference in the magnitude of the internal pool of available glucose and glutamine as well as the rate of conversion of glutamine and glucose into various amino acids. He supported the amino acid hypothesis according to which free amino acids are immediate precursors of proteins. Folkes

and Yemm (1958), Folkes (1959), Joy and Folkes (1965) also support

the amino acid hypothesis.

The data presented in table VI show that the amino acids

of reserve protein could supply all the amino acids except aspartate

for the synthesis of embryonic protein without much interconversion.

Moreover, the results with proline feeding to endosperms showed that

it could be translocated to the embryo and could be incorporated into

proteins as proline. Evidence from other experiments being reported

later in this thesis is also pertinent and presents additional

evidence for the amino acid hypothesis.

II.   Total Soluble Sugars During Germination

The changes observed in the total soluble sugars of the embryo

and the endosperm at various stages of development have been shown in

tables VII and VIII and fig 10 . In both lines the amounts of soluble

sugars present on any day in the embryo or endosperm were similar. In

the embryo prior to germination the amount present was very small and

it increased gradually, reaching a maximum on the 6th day in both the

lines. In the endosperm of the seed, a much higher amount of soluble

sugars was present and this increased gradually, reaching a maximum on

the 4th day of germination. In both the embryo and the endosperm the

maximum increase occurred between 2 and 4 days. A semi-quantitative

investigation using paper chromatography with n-butanol-pyridine-water

(Block et al., 1958) as a developing solvent revealed that in the

embryo of seeds prior to germination the most important sugars were

raffinose and sucrose in both lines. However, in the endosperm an

Table VII. Total soluble sugars of the embryo of etiolated barley
seedlings at various stages of development during
germination.

	mg/g seed	
days	Normal	Mutant
0	8.0	8.1
2	11.6	10.1
4	44.8	46.0
6	60.7	53.1
8	46.8	44.0
10	12.6	17.5

Table VIII. Total soluble sugars of the endosperm of etiolated barley
seedlings at various stages of development during
germination.

	mg/g seed	
Days	Normal	Mutant
0	36.4	36.3
2	40.6	39.9
4	80.6	76.9
6	38.0	40.8
8	5.7	10.0
10	2.6	2.7
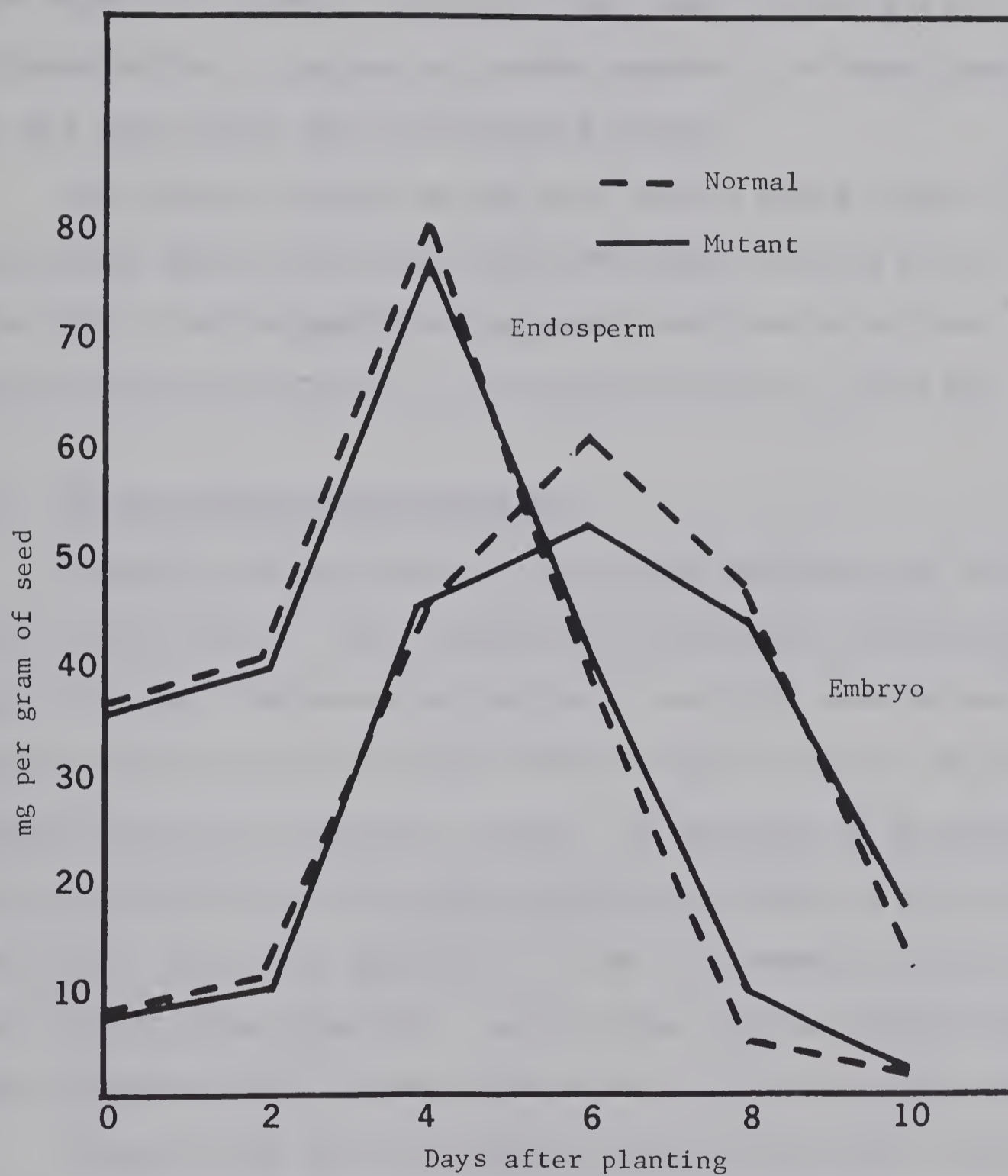
FIG. 10. Total soluble sugars of barley
endosperms and embryos during germination in the dark.

array of compounds including sucrose, raffinose and maltose were present. There were some unidentified compounds which from their Rf values appeared to be oligosaccharides. At 2 days the embryo also showed the presence of several other sugars including some oligosaccharides. Fructose and sucrose appeared to be major sugars at this stage rather than raffinose and sucrose.

The maximum increase in the total soluble sugars (table VII) and protein amino acids (table IV) in the embryo occurred at the same time (2 to 4 days) suggesting that intensive translocation of food material from the endosperm to the embryo took place at this time.

III. Organic Acids During Germination

Changes in organic acids of light grown seedlings were followed over a 10-day period. The seedlings were divided into shoots and roots plus endosperm. The determinations for 0- and 2-day material were made without the division of the plant material into two parts. The predominant acids were malate and citrate. In seedlings of the mutant there was more than twice as much malate as the normal at the 4-, 8- and 10-day stage (table IX, fig 11). The differences in citric acid were not very great (fig 12). In the normal citrate occurred in measurable amounts in seeds whereas in the mutant only traces were present.

Malate in the shoot was initially less in the mutant than the normal but reached values about 3 times those of the normal at the 8- and 10-day stage. Citrate was relatively low throughout and there was little difference between the two lines (table X). In the roots plus endosperm malate decreased throughout the period under

Table IX. Changes in organic acids at various stages of development
of barley seedlings grown in light.

| Days | mg/45 seedlings | | | |
| | Malic | | Citric | |
	Normal	Mutant	Normal	Mutant
0	0.5	0.5	1.2	T
2	T	0.5	T	0.5
4	10.6	20.6	1.2	2.0
6	24.6	19.9	3.8	2.8
8	16.4	44.2	1.6	2.6
10	36.8	100.3	2.1	1.8

T = Trace
Conditions of separation of organic acid esters by gas liquid chroma-
tography: 10% reoplex 400 on Gas-Chrom A 60-80 mesh, 5' x 1/8"
stainless steel column, oven temperature programmed from 75° to 175° at
a rate of 3.5^o per minute, injector temperature 200°, detector temperature
220°, hydrogen flame detector, hydrogen gas 25 ml per minute, carrier gas
nitrogen 25 ml per minute, recorder Beckman Model 1005, chart speed
0.5 inches per minute.

Table X. Changes in organic acids at various stages of development of
shoots of barley seedlings grown in light.

| Days | mg/45 shoots | | | |
| | Malic | | Citric | |
	Normal	Mutant	Normal	Mutant
0	N.D.	N.D.	N.D.	N.D.
2	N.D.	N.D.	N.D.	N.D.
4	5.1	3.6	-	-
6	23.2	14.6	0.8	0.6
8	15.5	40.4	0.7	1.2
10	30.2	96.9	1.2	1.8

N.D. = Not determined
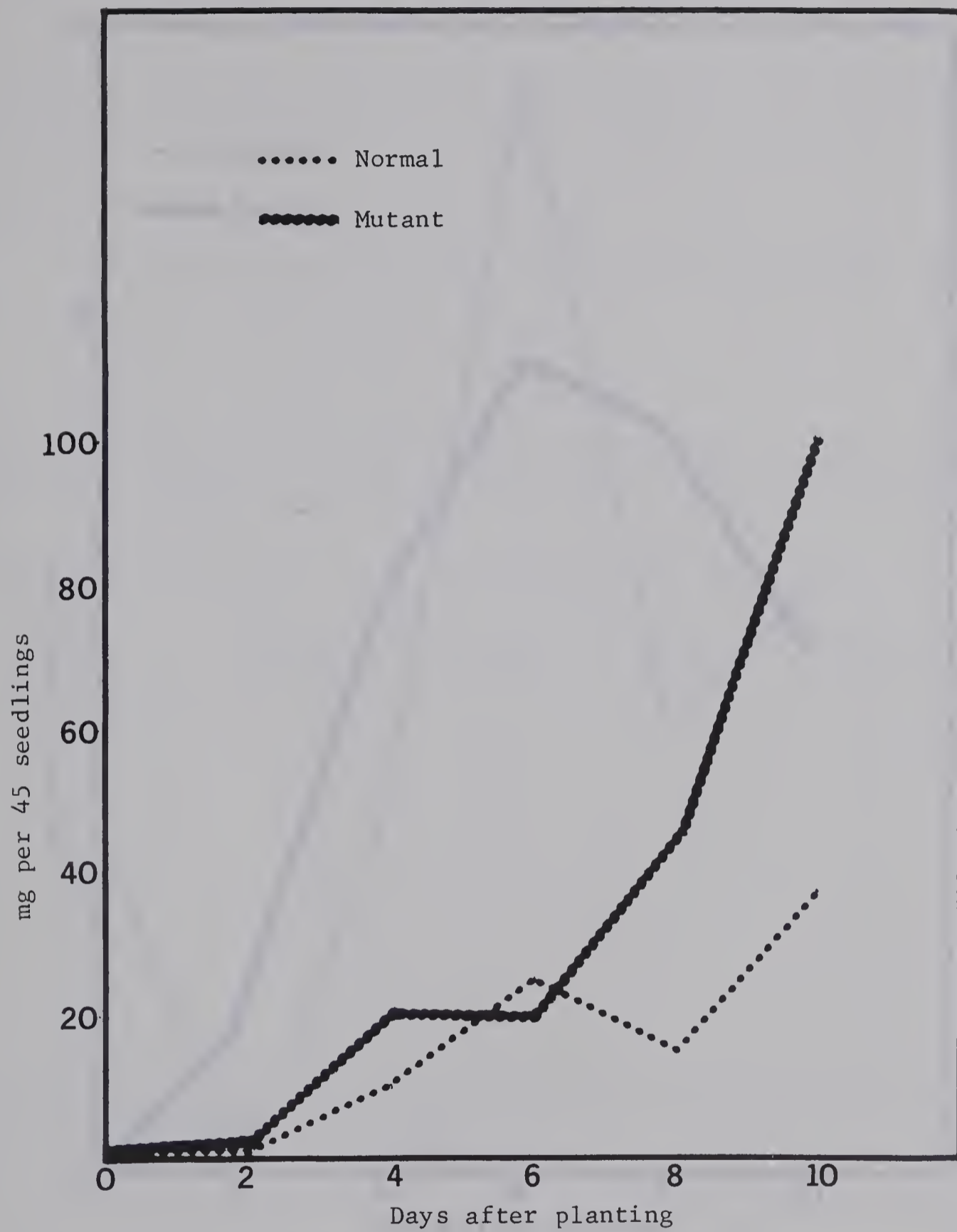
FIG. 11. Malic acid content of barley seedlings during germination.
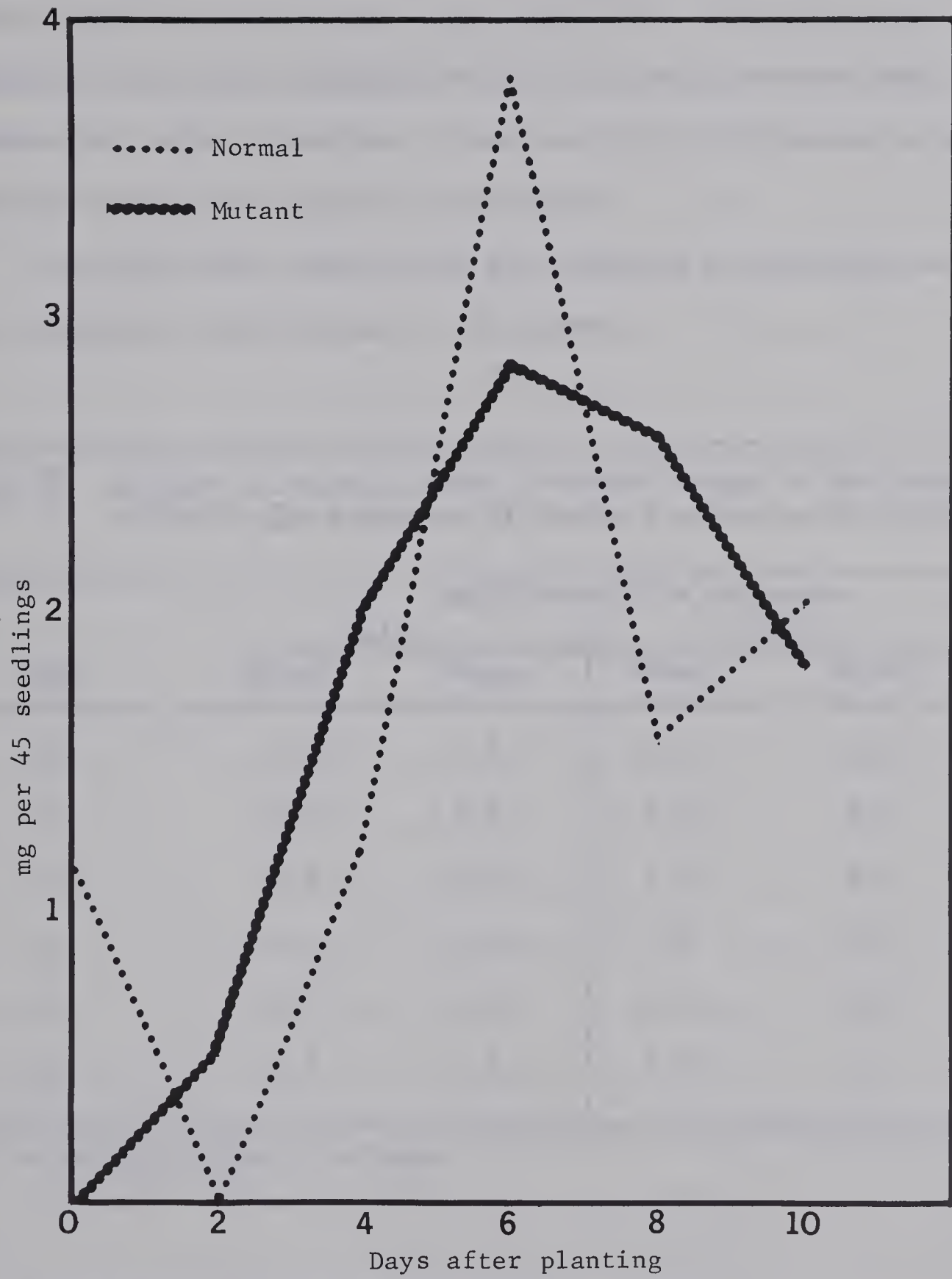
FIG. 12. Citric acid content of barley seedlings
during germination.

investigation in the mutant whereas in the normal at the 10-day

stage it showed a considerable rise (table XI). The citric acid

content of roots plus endosperm of both the lines increased until

6 days after which it declined. There was little difference in the

amount of citric acid present in the lines.

The only other organic acid that occurred in measurable amounts

was succinate at the 10th day in the shoots.

Table XI.   Changes in organic acids at various stages of development
            of roots plus endosperm of barley seedlings grown in light.

| | mg/45 roots plus endosperm | | | |
| | Malic | | Citric | |
Days	Normal	Mutant	Normal	Mutant
0	N.D.	N.D.	N.D.	N.D.
2	N.D.	N.D.	N.D.	N.D.
4	5.5	17.0	1.2	2.0
6	1.4	5.3	3.0	2.2
8	0.9	3.8	0.9	1.4
10	6.6	3.4	0.9	T

N.D. = Not determined, T = Trace

IV.   Amino Acid Composition of Chloroplast Protein

In order to find out if there were major differences in the

amino acid composition of the chloroplast protein of the normal and

the mutant the protein from chloroplasts purified on a discontinuous

sucrose density gradient was precipitated and its amino acid
composition determined. The results show that at 6 days the major
difference between the two lines was that the percent of leucine was
considerably higher in the mutant (table XII). At the 10-day stage
the major difference was that glutamic acid was higher in the mutant.
In both lines at 6 and 10 days the amino acids present in highest
amounts were glycine, leucine, alanine, glutamic and aspartic. In both
lines at 6 and 10 days histidine and tyrosine were very low. These
results are more similar to those given for lamellar protein by Weber
(Criddle, 1966) than for structural protein of chloroplasts given by
Criddle (1966).

If ammonia was included in calculating the percent distribution
of amino acids it was observed that in the mutant at both 6 and 10 days
its content was much higher than in the normal. The actual values for
the mutant were 16% and 25% ammonia at 6 and 10 days as against 13% and
11% for the normal. In considering these results it must be borne in
mind that the method used for purification of the chloroplast allowed
comparison of those that had achieved a certain range of density. The
fraction taken consisted of well developed chloroplast as seen in a
phase contrast microscope. As a consequence of this chloroplasts of
the mutant that had not fully developed were rejected. On the basis
of the results obtained it is not possible to conclude if there was
a qualitative difference in the chloroplast protein of two lines.

Table XII. Amino acid composition of protein of purified chloro-
            plasts from barley seedlings.

| | mole % amino acids | | | |
| | 6 days | | 10 days | |
Amino Acid	Normal	Mutant	Normal	Mutant
Lysine	5.5	5.7	4.5	5.3
Histidine	1.8	1.6	1.8	1.2
Arginine	5.0	4.6	4.3	3.9
Aspartic	9.0	8.5	8.9	9.5
Threonine	5.6	7.7	6.3	5.1
Serine	7.8	8.1	6.5	7.7
Glutamic	9.2	9.6	8.5	11.8
Proline	6.4	5.8	8.1	7.0
Glycine	10.4	9.8	10.6	10.9
Alanine	9.4	7.8	10.0	9.8
Valine	6.9	6.5	7.0	6.8
Isoleucine	4.9	4.4	5.0	4.5
Leucine	9.5	13.0	8.7	9.0
Tyrosine	3.2	2.5	3.6	1.8
Phenylalanine	5.4	4.3	6.3	5.7

V. Metabolism of Acetate-2-^{14}C by Barley Shoots

Since the cause of slow accumulation of chlorophyll in the
mutant could not be pin pointed by previous studies, it was decided
to look into the general pattern of metabolism of acetate. Because
acetate is a key metabolite it was the first labeled compound used
to detect differences in the metabolism between the normal and mutant
and further investigations were planned as an outcome of these studies.
The acetate metabolism studies were conducted on 6- and 10-day old
seedlings grown in light. The excised shoots were incubated in light
for 3 hours with an excess of acetate-2-^{14}C solution (10 μc/ml).

It may be seen that the greatest difference at 6 days was in
the percent label incorporated into the protein amino acids (table XIII).
In the normal 22% of the total label was incorporated into protein as
against only 7% in the mutant. The situation was in reverse in the
case of free amino acids. In the mutant free amino acids accounted
for 13% of the total label whereas in the normal they accounted for
only 8%. If the protein synthesizing efficiency is expressed as the
proportion of label in the free amino acids to protein amino acids,
for the normal the value was 2.8 as compared to 0.5 for the mutant. In
other words at this stage the normal was about 5 times more efficient
than the mutant in its protein synthesizing ability.

The percent of label incorporated into organic acids was more
in the mutant than in the normal. The difference in the percent
label incorporated in sugars and the ether soluble fractions by the
two lines was not great.

Table XIII. Percent distribution of label incorporated in different
            fractions of 6- and 10-day old barley shoots incubated
            for 3 hours in light with an excess of acetate-2-^{14}C
            solution (10 μc/ml).

Fraction	6 day		10 day	
	Normal	Mutant	Normal	Mutant
Ethanol soluble ether soluble	35	39	29	32
Ethanol insoluble ether soluble	10	8	18	16
Soluble sugars	1	1	2	2
Organic acids	24	32	29	31
Free amino acids	8	13	14	14
Protein amino acids	22	7	8	5
Total counts	1528516	1168337	1400017	1659389
Ratio of radioactivity in free a.a.: Protein a.a.	1:2.8	1:0.5	1:0.6	1:0.4

At the 10-day stage the major difference was still in the

amount of label incorporated into protein. In the normal 8% of the

total label was accounted for by protein as compared to 5% in the mutant.

The normal at this stage was only 1.5 times more efficient than the

mutant in its protein synthesizing activity. The difference in percent

incorporation of label in other fractions by the two lines was very

small. The smaller difference in the percent incorporation of label

into free and protein amino acids by the two lines at the 10-day stage

shows that the metabolism of the mutant seedlings and the normal

seedlings became alike as they grew older.

Since the major difference was in the label present in protein

amino acids it was decided to look into the distribution of label
in different protein amino acids. The results of this investigation
are shown in table XIV.

It may be seen that at the 6-day stage the percent incorporation
of label in leucine showed the greatest difference. In the normal
30% of the total label incorporated into protein amino acids was
accounted for by leucine as compared to only 7% in the mutant. Another
major difference at this stage was in the percent incorporation of
label in aspartic acid which in the mutant was 16% as compared to only
8% in the normal. The amino acid in which maximum label was incorporated
was glutamic acid in both the lines. There was no difference in the
percent label in proline, a minor difference in arginine and some what
greater difference in the other amino compounds.

At the 10-day stage leucine in the mutant accounted for 18% of
the total activity in protein as against 13% in the normal whereas
aspartic acid in the normal accounted for 8% of the total label in
protein as compared to 4% in the mutant. Glutamic acid in both lines
was the major amino acid accounting for over 57% of the total activity
present in protein amino acids. The difference in the percent label
present in arginine and proline was small and it was negligible in
the other amino acids. In general the difference in the normal and
the mutant with regard to percent distribution of label in different
amino acids at the 10-day stage was smaller than at the 6-day stage
confirming the previous conclusion that the metabolism of the mutant
approached the normal as the seedlings grew older.

Examination of the specific activities of different protein
amino acids (table XV) confirms the conclusions drawn previously.

Table XIV. Distribution of label in different protein amino acids as a percent of total label in this fraction in 6- and 10-day old barley shoots incubated for 3 hours in light with an excess of acetate-2-^{14}C solution (10 μc/ml).

Amino Acid	6 day		10 Day	
	Normal	Mutant	Normal	Mutant
Arginine	6	8	8	10
Aspartic	8	16	8	4
Glutamic	40	46	58	57
Proline	6	6	10	7
Leucine	30	7	13	18
Remainder	10	17	3	4

Table XV. Specific activities of protein amino acids of 6- and 10-day old barley shoots incubated for 3 hours in light with an excess of acetate-2-^{14}C solution (10 μc/ml).

	counts per micro mole			
	6 day		10 day	
Amino Acid	Normal	Mutant	Normal	Mutant
Arginine	93	115	61	90
Aspartic	70	116	40	14
Glutamic	318	323	300	186
Proline	85	77	87	35
Leucine	271	57	78	70

In addition it shows that at 10 days there was little difference
between the specific activity of leucine but there was considerable
difference in the specific activity of glutamic acid. In comparing
specific activities it must be remembered that the dilution of activity
due to endogenous amounts of amino acids present would be different for
the different lines.

VI. Metabolism of Glycine-2-^{14}C by Barley Shoots

Since glycine is one of the building blocks for the biosynthesis
of chlorophyll its metabolism in the two lines was compared. The
studies were conducted on 6-day old shoots excised from seedlings grown
in light. They were incubated with 2.5 μc of glycine-2-^{14}C in light
in a fume hood. The label was taken up by the shoots in 40 to 50 minutes
after which distilled water was added. Analyses of the shoots were
conducted after 1, 2 and 3 hours. The purity of the labeled glycine
was found to be at least 99.9% by simultaneous chromatographic and radio-
activity analyses. The percent distribution of incorporated label
into different fractions was determined and has been shown in table XVI.

It can be seen that at the end of 1 hour more than 56% of the
label was in free amino acids and it decreased with time in both lines.
This decrease was more pronounced in the normal than in the mutant.
This decrease of label in free amino acids was accompanied by an in-
crease of label in protein amino acids. The changes in the percent
incorporation of label with time into different fractions in both
lines were similar. The higher incorporation of label in the ethanol
soluble ether soluble fraction in the normal after 2 hours may have

Table XVI. Percent distribution of label incorporated in different fractions of 6-day old barley shoots incubated with glycine-2-^{14}C in light.

Fraction / Time in hours	Normal			Mutant		
	1	2	3	1	2	3
Ethanol soluble ether soluble	6	9	10	5	5	8
Ethanol insoluble ether soluble	T	1	1	T	1	1
Soluble sugars	2	2	3	1	3	2
Organic acids	7	9	9	6	8	7
Free amino acids	56	39	32	58	46	40
Protein amino acids	29	40	45	30	37	42
Total counts	235672	264940	287195	184180	247779	275101
Chlorophylls	4	5	4	3	4	3

T = Trace

been due to more utilization of glycine-2-^{14}C for the synthesis of chlorophyll and its intermediates. The ethanol insoluble ether soluble fraction accounted for a very minor portion of the total label. There was little difference in the amount of incorporation of label into sugars and organic acids between the mutant and the normal. In general it may be concluded that the normal and the mutant utilized glycine-2-^{14}C in a similar manner.

The distribution of label into different free amino acids as a percent of total label incorporated into free amino acids was determined.

It may be seen (table XVII) that the maximum label was incorporated
into serine in both lines. Glycine itself accounted for only 8 to 12%
in the normal and only 11 to 17% in the mutant. Apparently soon after
uptake of glycine it was converted mostly to serine. In both lines
the percent label in glycine increased at the end of 2 hours as
compared to 1 hour. However, these changes may merely be a reflection
of changes in the level of label in serine. In the normal alanine had
more label in the first 2 hours than in the mutant. Aspartate and
lysine acquired low levels of label.

Table XVIII shows the percent distribution of label in different
protein amino acids. In both lines glycine and serine together
accounted for over 90% of the total activity. The difference between
the two lines with respect to percent distribution of label in dif-
ferent protein amino acids at the end of 2 and 3 hours was
negligible. It is interesting to note that serine which accounted
for over 70% of label in free amino acids never accounted for more
than 48% of the total label in protein amino acids. As compared to
this glycine which accounted for less than 17% of the total label in
free amino acids accounted for more than 50% in the protein fraction.
Alanine which had similar activity in the free amino acid fraction
as glutamic acid was incorporated much more efficiently in protein
at the end of 3 hours. It was surprising to note that glutamic acid
which acquired label in the 1st hour was not incorporated into protein
until 3 hours. Even at the end of 3 hours it was very weakly
labeled.

Table XVII. Distribution of label in free amino acids as a percent of total label in this fraction in 6-day old barley shoots incubated in light with glycine-2-^{14}C.

Time in hours Amino Acid	Normal			Mutant		
	1	2	3	1	2	3
Aspartic	1	1	1	T	1	2
Serine	81	74	77	77	67	71
Glutamic	3	3	3	2	3	3
Glycine	8	12	12	13	17	11
Alanine	3	4	2	2	2	3
Lysine	1	3	1	1	3	2
Basic Compound A*	1	1	2	2	3	3
Remainder	2	2	2	3	4	5

T = Trace

*Basic compound A = A basic compound eluted on the short column of the amino acid analyzer at a position where ethanolamine is eluted. This compound on the basis of its position of elution has been tentatively identified as ethanolamine.

Table XVIII. Distribution of label in protein amino acids as a percent of total label present in this fraction in 6-day old barley shoots incubated in light with glycine-2-^{14}C.

Time in hours Amino Acid	Normal			Mutant		
	1	2	3	1	2	3
Serine	41	46	43	38	48	42
Glutamic	-	-	T	-	-	T
Glycine	54	52	50	61	50	52
Alanine	3	1	3	1	1	3
Remainder	2	1	4	-	1	3

-80-

T = Trace

Discussion

The pattern of distribution of glycine-2-^{14}C in different fractions has been given in table XVI. As mentioned earlier only label retained in protein amino acids of the pellet fraction has been accounted for. The label in other insoluble material was disregarded as the total activity observed for the pellet was mostly present in protein amino acids. The general pattern of distribution of label in organic acids, sugars and free amino acids observed agrees well with the results obtained by Sinha and Cossins (1964). The synthesis of sugars from glycine may have occurred by the pathway suggested by Wang and Waygood (1962). The labeling of organic acids could occur via glyoxylate produced by the transamination reaction from glycine (Sinha and Cossins, 1964). The label present in the ether soluble fraction at the end of 3 hours was about 10%. This value is high when compared to the value of less than 2% observed by Sinha and Cossins (1964). The reasons for the difference may be the type of plant material used. In their studies non-photosynthetic tissue slices were used. In the present experiment photosynthesizing barley leaves have been used and these leaves incorporated 3 to 5% of the total activity retained by the plant into chlorophylls. This to some extent was responsible for a higher proportion of label in the ether soluble fraction. Incorporation of glycine-2-^{14}C into chlorophylls in photo-synthesizing organisms has been shown by Della Rosa et al. (1953), Wang and Waygood (1960), Perkins and Roberts (1960) and Brezeski and Rucker (1960). This incorporation takes place via ALA.

More than 76% of the activity was present in free and protein

amino acids. Of the activity in free amino acids over 70% was in serine

(table XVII). This value is very high compared to only 34% in serine

obtained by Wang and Burris (1963) after 45 minutes. Glycine apparent-

ly was converted to serine at a very rapid rate. Formation of serine

from glycine has been shown in plant tissues by Wang and Waygood (1962),

Wang and Burris (1963, 1965a), Sinha and Cossins (1964), Miller and

Schmidt (1965) and Cossins and Sinha (1966). The pathway for such con-

version has also been demonstrated. In their studies Wang and Burris

(1963) observed that only 10% of the activity present in free amino

acids was retained in glycine at the end of 45 minutes. This compares

very well with 8% of the activity present in glycine at the end of 1

hour in this study. A comparison of label in glycine in protein amino

acids (table XVIII) and free amino acid (table XVII) showed that in

spite of there being much less activity present in glycine in the free

amino acid fraction its proportion in protein was always higher than

serine. If the specific activity of glycine in free amino acids was

extremely high as compared to serine due to a higher endogenous pool

of serine such results could be obtained. The amount of glycine present

in protein was not much different than serine and although the endogenous

pool of free serine was always higher than glycine the specific activity

of serine was always higher than glycine in free amino acids (table XIX).

The specific activity of serine in the protein fraction was always

lower than glycine (table XX). This suggests that serine was present

in 2 pools and the 1 which was less labeled was easily accessible

for protein synthesis. It has been shown that serine formation could

occur by 2 different pathways (Bassham, 1965):

Table XIX. Specific activities of free amino acids of 6-day old barley shoots incubated in light with glycine-2-^{14}C.

Time in hours Amino Acid	Counts per micro mole in thousands					
	Normal			Mutant		
	1	2	3	1	2	3
Serine	32.77	18.60	12.22	5.50	6.87	7.64
Glutamic	0.35	0.27	0.28	0.05	0.09	0.11
Glycine	7.67	8.55	10.92	4.37	5.97	3.85
Alanine	0.66	0.40	0.31	0.13	0.14	0.18

Table XX. Specific activities of protein amino acids of 6-day old barley shoots incubated with glycine-2-^{14}C in light.

Time in hours Amino Acid	Counts per micro mole in thousands					
	Normal			Mutant		
	1	2	3	1	2	3
Serine	0.54	0.76	0.85	0.41	0.80	0.87
Glutamic	-	-	T	-	-	T
Glycine	0.78	1.05	1.17	0.84	1.02	1.46
Alanine	0.04	0.02	0.06	0.01	0.02	0.07

T = Trace

(a)   Formation by transamination of hydroxypyruvic acid

$$\begin{array}{c} CH_2OH \\ | \\ C=O \\ | \\ CO_2^- \end{array} \xrightarrow{\text{glutamic acid}} \begin{array}{c} CH_2OH \\ | \\ HCNH_2 \\ | \\ CO_2^- \end{array} + \ \alpha\text{-ketoglutaric acid}$$
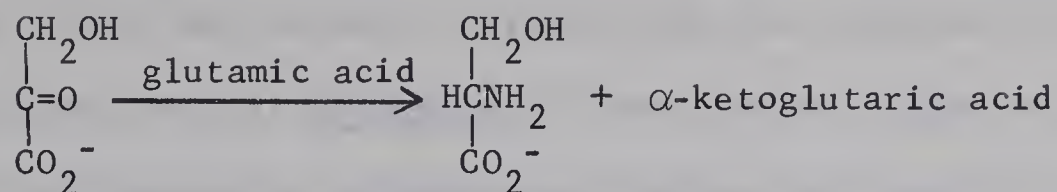
(b)   Hydroxymethylation of glycine by hydroxymethyl-
       tetrahydrofolic acid

$$\begin{array}{c} H_2C-NH_2 \\ | \\ CO_2^- \end{array} + \ THFA-CH_2OH \longrightarrow THFA \ + \begin{array}{c} CH_2OH \\ | \\ HCNH_2 \\ | \\ CO_2^- \end{array}$$
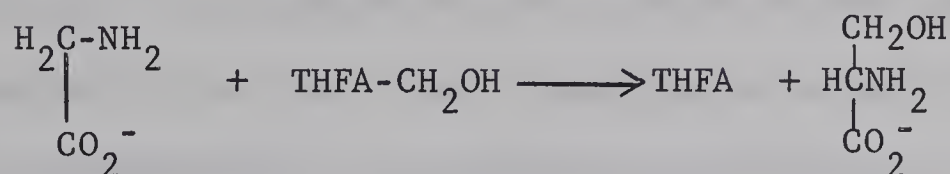
Probably both routes were operative and the pathway producing less

radioactive serine contributed more serine for protein synthesis.

Besides glycine and serine, alanine, glutamate, lysine and

asparate also acquired label. Label in alanine could occur by trans-

amination of pyruvic acid which must have originated from glycine. In

the experiments conducted by Sinha and Cossins (1964) the pyruvate

fraction was labeled and possessed 9% of the total label present in

organic acids of carrot tissues. Glutamate could occur by two path-

ways: (a) by entry of carbon from glycine in the TCA cycle followed by

transamination of α-ketoglutaric acid, and (b) by deamination of ALA

formed from glycine and succinate to yield ketoglutaraldehyde which on

oxidation would give rise to α-ketoglutaric acid (Shemin succinate-

glycine cycle). This then could undergo transamination to yield

glutamate. Although glutamate acquired label in the 1st hour, the

label did not appear in glutamate of protein until 3 hours. Even then

the labeling of protein glutamate was very weak. This suggests that like

serine, glutamic acid may have been present in 2   separate pools.

Smith et al. (1961) had obtained evidence for the presence of separate

pools of glutamic acid in Chlorella. It was further suggested that the

active pool was located in chloroplasts whereas the inactive pool

was located outside of chloroplasts. The labeled glutamate produced

by either of the pathways mentioned above was removed from the site of

protein synthesis. As opposed to this alanine became labeled in the

protein fraction in the 1st hour. Labeling of asparate may have

occurred via transamination of oxaloacetic acid which in turn may have

acquired label from labeled carbon of glycine entering the TCA cycle.

Basic compound A (table XVII) which acquired label in the 1st

hour was eluted on the short column of the amino acid analyzer. From

the position of its elution it has been tentatively identified as

ethanolamine. Ethanolamine could originate from labeled serine on

decarboxylation (Elwyn et al., 1955). An examination of the specific

activity of serine and glycine in free amino acids in the normal shows

that the specific activity of serine decreased with time and was

accompanied by an increase in the specific activity of glycine. In view

of the fact that all the labeled glycine was taken up by the shoots in

the first hour and no further labeled glycine was added it appears that

serine was converted back to glycine. A similar resynthesis of glycine

was suggested by the results of Sinha and Cossins (1964) and they

suggested that this could occur via ethanolamine. The data on specific

activities and the occurrence of measurable amounts of the compound

tentatively identified as ethanolamine lends support to their

suggestion.

The specific activity data for the free amino acid fraction of the mutant was different than for the normal. The specific activities of the 3 amino acids other than glycine increased with time. In the normal only the specific activity of glycine increased whereas that of the other 3 amino acids decreased. The differences in the specific activities of free amino acids in the normal and mutant can be explained on the basis of the 2 to 3 fold higher endogenous amounts of free amino acids present in the mutant.

The specific activity data for amino acids in the protein fraction of both the normal and the mutant show a gradual increase in each of serine glycine and alanine. This must have been due to continuing incorporation of labeled amino acids from the free pool. This lends additional support to the "amino acid" hypothesis of protein synthesis discussed earlier.

VII. Metabolism of Leucine-U-^{14}C by Barley Shoots

The studies on the metabolism of acetate-2-^{14}C referred to earlier showed that at the 6-day stage there was a considerably higher incorporation of label into protein by the normal. In order to determine if this was really a consequence of a higher rate of protein synthesis in the normal, studies on the metabolism of leucine were undertaken as leucine is known to be incorporated into protein directly. The shoots excised from the seedlings grown in light for 6 days were incubated in light with leucine-U-^{14}C (2.5 μc), the purity of which was found to be at least 99.9% by simultaneous chromatographic and radioactivity analyses. The shoots took up all the label in 40 minutes

after which distilled water was added. The analyses were conducted
at the end of 1, 2 and 3 hour periods.

From table XXI it is seen that the percent label incorporated
into amino acids in the normal was considerably higher at the end
of the 1st hour than in the mutant. Even at the end of 3 hours
the percent incorporation of label in protein amino acids in the
mutant was less than the normal. In the normal there was little change
in the percent label accounted for by the protein over a 3 hour
period presumably because it had such a high rate of protein synthesis
that most of the label taken up was incorporated into protein in the
1st hour. In the mutant this was not the case and the percent in-
corporated label at the end of 2 hours rose to 73% from 67% and
then remained constant. The situation was reversed for the free amino
acids which accounted for less label in the normal at any stage than
in the mutant. These results are in conformity with the previous
observations made with the studies on the metabolism of acetate-2-^{14}C.
If the protein synthesizing efficiency is expressed as the proportion
of label in free amino acids to protein amino acids the normal was 2 to
3 times more efficient than the mutant. Of the remaining fractions
organic acids constituted about 5 to 7% of the total activity after
2 hours in both lines. The ethanol soluble ether soluble fraction in
the normal accounted for 2% of the total activity at any time whereas in
the mutant its contribution increased from 3% to 5% during the in-
cubation period. There was negligible label present in sugars or
the ethanol insoluble ether soluble fraction in both lines. More
than 90% of the label accounted for was retained in free and protein
amino acid fractions.

Table XXI. Percent distribution of label incorporated in different fractions of 6-day old barley shoots incubated with leucine-U-^{14}C in light.

	Normal			Mutant		
Time in hours	1	2	3	1	2	3
Fraction						
Ethanol soluble ether soluble	2	2	2	3	4	5
Ethanol insoluble ether soluble	-	-	1	-	1	1
Soluble sugars	1	1	-	1	-	-
Organic acids	3	6	5	5	5	7
Free amino acids	10	7	9	24	17	14
Protein amino acids	84	84	83	67	73	73
Total counts	337400	322200	322000	169200	253100	271100
Ratio of radioactivity in free a.a.: Protein a.a.	1:8	1:12	1:9	1:3	1:4	1:5

The distribution of label in different free amino acids as a percent of total label incorporated in this fraction is given in table XXII. It is seen that almost 90% of label present in free amino acids was accounted for by leucine, the difference between the two lines being negligible. Of the other amino acids glutamic, proline and glycine together contained between 3 to 6% of the label in both lines. In the mutant arginine and an unknown X were very weakly labeled as compared to the normal in which they together accounted for 3 to 5% of the total label in free amino acids. In both lines the percent label incorporated into aspartate and other amino acids was very low.

Table XXII. Distribution of label in free amino acids as a percent
of total label in this fraction in 6-day old barley
shoots incubated with leucine-U-^{14}C in light.

Time in hours Amino Acid	Normal 1	2	3	Mutant 1	2	3
Aspartic	-	-	-	1	-	-
Glutamic	1	3	3	2	3	3
Proline	1	2	1	-	1	1
Glycine	1	1	1	1	1	1
Leucine	93	86	88	94	93	93
Arginine	2	3	2	T	T	T
Unknown X	1	2	2	T	T	T
Unknown Y	-	1	1	1	1	1
Remainder	1	2	2	1	1	1

T = Trace

Unknown X - A basic compound eluted after ammonia but before arginine
on the short column.

Unknown Y - A non-amino compound eluted after proline but before
glycine on the long column.

In the protein fraction essentially all of the label was present
in leucine in both the lines. Glutamate, glycine and arginine contained
traces of label.

When acetate-2-^{14}C and leucine-U-^{14}C were fed to the shoots of
the normal and the mutant in light the protein synthesizing activity
of the normal was higher. In order to determine if protein synthesis

in the normal was also more efficient in the dark leucine-U-^{14}C was fed in the dark to the shoots excised from the dark grown seedlings. The experiment was carried out under similar conditions as for the shoots fed in light. The percent distribution of label in different fractions at the end of 2 hours is shown in table XXIII.

It is interesting to note that the two lines did not differ much in the metabolism of leucine in the dark. In fact the mutant was slightly superior to the normal with regard to the incorporation of label into protein amino acids. Also as might be expected the protein synthesizing capacity of both lines was poorer in the dark than in the light (tables XXIII, XXI). In both light and dark about 90% of the activity was retained in the free and protein amino acid fractions at the end of 2 hours. The percent incorporation of label in other fractions in light and in dark was similar in both lines.

The distribution of label as a percent of label incorporated in free amino acids is shown in table XXIV. Over 90% of the activity was accounted for by leucine in both lines. The amino acids aspartic, glutamic, glycine and proline also acquired some label in both lines. The distribution of label in protein amino acids showed that in both lines all the activity was accounted for by leucine. The pattern of distribution of leucine did not seem to be affected by light.

Discussion

Labeled leucine has been used to study the synthesis of protein in plant and animal systems since it is directly incorporated into protein. In both lines practically all the activity present in protein

Table XXIII. Percent distribution of label incorporated in different fractions of 6-day old etiolated barley shoots incubated with leucine-U-^{14}C for 2 hours in the dark.

Fraction	Normal	Mutant
Ethanol soluble ether soluble	3	2
Ethanol insoluble ether soluble	-	1
Soluble sugars	2	2
Organic acids	4	4
Free amino acids	53	48
Protein amino acids	38	43

Table XXIV. Distribution of label in free amino acids as a percent of total label in this fraction in 6-day old etiolated barley shoots incubated with leucine-U-^{14}C for 2 hours in the dark.

Amino Acid	Normal	Mutant
Aspartic	T	1
Glutamic	1	2
Proline	T	1
Glycine	1	1
Leucine	96	93
Remainder	2	2

T = Trace

after leucine-U-^{14}C feeding was accounted for by leucine. This is in

agreement with the results obtained by Splittstoesser (1967) who work-

ing on leucine-U-^{14}C metabolism in maize noted that the label incorporated

into insoluble residue was present in leucine only. In free amino acids,

however, this was not the case as leucine accounted for only 86% to 94%

of the total activity present in free amino acids depending upon the

time of incubation (table XXII). This is in close agreement with the

results of Splittstoesser (1967). Among other amino compounds glutamic

acid, arginine, proline and glycine acquired label. The specific

activity data presented in table XXV show that the specific activity

of leucine was very high as compared to any other amino acid. Of the

remaining acids arginine had the highest specific activity. In the

normal, for example, the specific activity of arginine was 20 times

higher than glutamate at the end of 1 hour and 10 times higher at

the end of 3 hours. The synthesis of arginine in plants proceeds

via glutamic acid. The only additional carbon in arginine comes from

carbamyl phosphate. Synthesis of carbamyl phosphate takes place in

the presence of CO_2, NH_3 and ATP. The higher specific activity in

arginine as compared to glutamate on the basis of the pathway of

arginine biosynthesis (White et al., 1964) must come from carbamyl

phosphate. This means that CO_2 involved in the synthesis of

carbamyl phosphate should have originated from leucine and the carbamyl

phosphate synthesized should be highly radioactive. The degradation

of leucine gives rise to acetyl CoA and acetoacetate (Meister, 1965).

Of this acetyl CoA can give rise to labeled CO_2 via the TCA cycle.

Evidence from the present study supporting this was the considerable

Table XXV. Specific activity of free amino acids of 6-day old barley shoots incubated with leucine-U-^{14}C in light.

	Counts per micro mole in thousands					
	Normal			Mutant		
Time in hours	1	2	3	1	2	3
Amino Acid						
Glutamic	0.24	0.23	0.35	0.16	0.16	0.17
Leucine	142.43	71.73	81.63	78.84	37.31	41.14
Arginine	4.79	3.54	3.45	0.78	0.62	0.18

Table XXVI. Specific activity of leucine in the protein fraction of 6-day old barley shoots incubated with leucine-U-^{14}C in light.

| | Counts per micro mole in thousands | | | | | |
| | Normal | | | Mutant | | |
Time in hours	1	2	3	1	2	3
Leucine	13.70	12.10	13.88	9.41	14.95	13.16

Table XXVII. Specific activity of leucine of 6-day old etiolated barley shoots incubated with leucine-U-^{14}C for 2 hours in the dark.

| | Counts per micro mole in thousands | | | |
| | Free amino acids | | Protein amino acids | |
	Normal	Mutant	Normal	Mutant
Leucine	53.51	58.00	6.23	7.37

activity present in organic acids (table XXI). The major activity of arginine must come from this CO_2 and degradation of arginine should show a higher label in the amidine carbon of arginine. Alternately CO_2 could arise directly from leucine by decarboxylation. The enzyme L-valine carboxylase can act on L-leucine (Dixon and Webb, 1964) and liberate labeled CO_2.

On account of different endogenous amounts of leucine present in protein and free amino acids of the normal and mutant direct comparisons of specific activities could not be made. The amount of leucine in which the normal and the mutant shoots were incubated was only about 0.01 µmole and this is negligible compared to the endogenous amounts of free leucine present in both lines. As a result the distribution of label may depict the usual mode of leucine utilization in both lines.

The activity incorporated into the organic acid fraction (table XXI) could be due to the entry into the TCA cycle of acetate originating from the degradation of leucine (Meister, 1965). The activity incorporated in the ether soluble fraction may be due to isoprenoids synthesized from acetoacetate and acetate derived from leucine degradation. The specific activity of leucine in protein of shoots incubated in light was 2 times greater than the specific activity of leucine in protein of shoots incubated in dark (tables XXVI and XXVII). The higher protein synthesizing ability of light grown shoots in light is probably due to protein synthesis by chloroplasts. Chloroplasts have been shown to synthesize protein by Eisenstadt and Brawerman (1964), Spencer and Wildman (1964), Goffeau and Brachet (1965),

Spencer (1965) and Trachsel (1967).

VIII. <u>Effect of δ-aminolevulinic Acid and Glycine Feeding on Chlorophyll Synthesis</u>

It has been suggested by several workers that in higher plants one control of chlorophyll biosynthesis is exerted at the 1st step namely the synthesis of ALA from succinate and glycine. The behavior of the mutant indicated that some step in the biosynthesis of chlorophyll was quantitatively affected. One possibility was that the synthesis of ALA might have been slower in light in the mutant than in the normal. Two experiments, one using glycine-2-^{14}C and ALA-4-^{14}C and the other using unlabeled ALA were conducted.

Before excising the shoots, the dark grown seedlings were exposed to light for 1 hour to overcome the lag phase in chlorophyll synthesis. The excised shoots were incubated for 3 hours in light with glycine-2-^{14}C (2.5 μc) or ALA-4-^{14}C (2.5 μc). The shoots took up the solution in 40 to 50 minutes after which distilled water was added. The chlorophyll determinations and the measurement of total activity shown in table XXVIII were done at the end of 3 hours.

It is seen that the incorporation of label into chlorophyll was different in the two lines for both glycine and ALA. The lower value in the normal for label incorporated from glycine-2-^{14}C may be due to more diversion of glycine to other biosynthetic pathways in this line. There was a considerably higher incorporation of label from ALA than glycine into chlorophylls in both lines. The incorporation of label from ALA into chlorophyll in the normal was higher than in the mutant.

Table XXVIII. Total and specific activity of chlorophylls of 6-day
old etiolated barley shoots incubated in light for
3 hours with glycine-2-^{14}C and δ-aminolevulinic acid-
4-^{14}C.

Incubation with	Chlorophyll content in μg		Total Counts		Specific activity in counts per μg of chlorophyll	
	N	M	N	M	N	M
Glycine-2-^{14}C	180	110	13800	18350	77	167
δ-ALA-4-^{14}C	170	120	342850	287650	2017	2397

N = Normal, M = Mutant
Figures are averages of two replicates

Since label was fed for a limited time the dilution of the activity due
to differences in total chlorophyll content as reflected in the specific
activities, would be different for the normal and the mutant. The
change in the chlorophyll content within a line as a result of in-
cubation with glycine or ALA was negligible. Comparison of the specific
activity of chlorophyll as a result of incubation with ALA and glycine
showed that the specific activity in the normal increased from 77 counts
per microgram to 2017 counts per microgram whereas in the mutant it in-
creased from 167 counts per microgram to 2397 counts per microgram.
This represented an increase of 26 fold in the normal as against 14
fold in the mutant.

In the second experiment 6-day old seedlings grown in dark
were exposed to light for 1 hour as in the previous experiment. The

shoots from these seedlings were excised and incubated with an excess

of 0.05 M ALA in light for 3 hours. Shoots incubated in distilled

water for the same period constituted a control. The chlorophyll

concentrations were determined prior to incubation and after 3 hours

of incubation. The results have been expressed as the amount of

chlorophyll synthesized during the period of incubation.

It may be seen that the amount of chlorophyll accumulated was

greatest in both lines when the shoots were incubated with an excess

of ALA (table XXIX). In the normal the chlorophyll synthesized in

presence of ALA was 94 µg whereas in the mutant it was 63 µg. The in-

crease in chlorophyll due to incubation with ALA as compared to distilled

water in the normal was 32 µg whereas in the mutant it was 22 µg.

Although there was about 50% increase in the amount of chlorophyll

synthesized due to ALA over the distilled water control in both lines,

the mutant did not synthesize as much chlorophyll as the normal during

the incubation period.

Table XXIX. Chlorophyll synthesized by 6-day old etiolated barley
             shoots incubated with ALA and distilled water in
             light for 3 hours.

| Incubation with | µg chlorophyll/g fresh wt | |
	Normal	Mutant
0.05 M ALA	94	63
Distilled Water	62	41

Figures are averages of two replicates.

GENERAL DISCUSSION AND CONCLUSIONS

Although some discussion followed the descriptions of individual experiments this general discussion will attempt to summarize the findings of various aspects of the study.

The studies by Maclachlan (1962) and Miller (1965) raised the possibility of a difference in the food reserves of the seeds of Gateway barley and the mutant, hence analyses of endosperm and embryo of etiolated seedlings to determine their contents of free and protein amino acids and total soluble sugars were conducted. There were no differences between the normal and the mutant in the nitrogenous substances in the embryo. The amount of glycine, which is the nitrogenous precursor of chlorophyll, was similar in the free and protein fractions of the embryo of both lines. The analysis of endosperm protein showed higher levels in the normal of most amino acids particularly glutamic and proline. Thus the normal had a higher amount of reserve nitrogen. However the fact that in the shoots, where synthesis of chlorophyll takes place, the level of glycine was the same in both lines indicated that the higher reserve of nitrogen in the normal might not have been responsible for the observed differences between the two lines. The amounts of total soluble sugars were not different between the two lines showing that the supply of carbon through this source was not different. This together with the observation that the level of free glycine in light grown seedlings of the mutant was greater than in the normal indicated that there was no scarcity of chlorophyll building blocks in the mutant.

In light grown shoots a considerably higher accumulation of malate was observed (table X) for the mutant than the normal. Whether a high level of malate could inhibit some enzyme involved in the biosynthesis of chlorophyll is not known. However, there have been no reports of inhibition of chlorophyll synthesis by malate and the accumulation of malate may have been the result of lesser utilization of succinate for chlorophyll synthesis.

Electron microscopic studies by Maclachlan and Zalik (1963) had shown that the chloroplasts of the mutant seedlings in early stages of development contained large vesicles but no normal lamellae or grana. This raised the possibility of a particular protein being present in lower amounts in the chloroplasts of the mutant. Therefore, the amino acid composition of protein of purified chloroplasts isolated from the 6- and 10-day old seedlings of the normal and the mutant was examined. The amino acid composition of the chloroplast protein was similar for the two lines but the similarity may have arisen in part as a consequence of the limitation of the method of chloroplast purification discussed earlier.

In order to determine which step in the biosynthesis of chlorophyll was quantitatively affected, the general pattern of acetate metabolism in light in both lines was examined. Since most biosynthetic pathways can start from acetate, the magnitude of diversion of acetate carbon into different pathways in the two lines was investigated. The studies showed that the two lines mainly differed in their incorporation of acetate into free and protein amino acids particularly at the 6-day stage. In the normal considerable activity was incorporated into protein

whereas in the mutant free amino acids retained more of the activity. The difference between the normal and the mutant at the 10-day stage was much less. These results parallel the chlorophyll accumulation studies from which it was found that the difference in the chlorophyll content of leaves was greater at 6 days than at 10 days. Thus it appeared that the higher chlorophyll synthesis in the normal might be linked with higher protein synthesis.

The results in table XIII showed that the differences observed between the normal and the mutant could not be attributed to the differences in the fixation of $^{14}CO_2$ by photosynthetic reactions, although $^{14}CO_2$ may have been evolved and utilized for photosynthesis to some extent. The percent distribution of label in protein amino acids at the 6-day stage showed that in the normal considerable synthesis of leucine had taken place and there have been reports of correction of chlorophyll synthesis of some mutants by the addition of leucine (Walles, 1967). However, an experiment with leucine feeding to the seedlings of the mutant showed that it did not correct the chlorophyll deficiency. Moreover, analysis of the shoots of the mutant for free amino acids in the experiments involving glycine and leucine feeding showed that in fact the amounts of all free amino acids including leucine were considerably higher in the mutant grown in light. This indicated that of the free amino acids analyzed there was no scarcity for the synthesis of protein but the protein synthesizing capacity of the mutant was impaired.

Since glycine is the nitrogenous building block for chlorophyll biosynthesis, its metabolism by the shoots excised from 6-day old light

grown seedlings of both lines was examined. The two lines did not differ much from each other in their pattern of glycine metabolism. Feeding leucine-U-^{14}C to shoots excised from 6-day old light grown seedlings showed that the normal was more efficient in the synthesis of protein in light. On the other hand feeding leucine in the dark to etiolated shoots confirmed that the two lines did not differ in their protein synthesis. This is in agreement with the finding that chlorophyll mutants accumulate large quantities of free amino acids when grown in light (Veleminsky et al., 1963 and Faludi-Daniel et al., 1965).

Comparison of leucine metabolism in light and dark suggested that the synthesis of protein was stimulated to a greater degree by light in the normal than in the mutant. The studies with inhibitors by Margulies (1962, 1967), Gassman and Bogorad (1965, 1967a) and Kirk and Allen (1965) have shown that inhibition of protein synthesis resulted in a lower accumulation of chlorophyll. The Gateway mutant under study had a lower rate of protein synthesis in light and this lower rate of protein synthesis caused slower accumulation of chlorophyll. Under light conditions the lower synthesis of protein could be in the chloroplasts or in the cytoplasm or both. On the basis of studies of Anderson and Smillie (1966) and Aaronson et al. (1967) the inhibition of chlorophyll synthesis by chloramphenicol observed by different workers was probably due to inhibition of protein synthesis in the chloroplasts. Gassman and Bogorad (1967a) have shown that the inhibition of chlorophyll synthesis by chloramphenicol in rapidly greening bean leaves could be partially overcome by ALA while Kirk and Allen (1965) proposed

that the greening was controlled by the synthesis of protochloro-
phyllide holochrome and that protein synthesis was needed for the
production of the holochrome. In view of this it was decided to
investigate whether synthesis of ALA was limiting in the mutant.

If ALA synthesis was slower in the mutant then the increase in
the specific activity of the chlorophyll from ALA feeding as compared
to glycine feeding should have been greater in the mutant than in the
normal. The results in table XXVIII showed that there was a 26 fold
increase in the specific activity of chlorophyll in the normal from ALA
feeding over glycine feeding whereas in the mutant this increase was
only 14 fold. This indicated that probably a slower rate of synthesis
of ALA was not responsible for slow accumulation of chlorophyll in the
mutant. As the amount of ALA or glycine fed in this experiment was
limited the increase in chlorophyll synthesis due to ALA feeding could
not be determined. In fact there was little difference in chlorophyll
synthesized when either of the compounds was fed. Therefore, shoots
excised from etiolated seedlings were incubated with an excess of
0.05 M ALA solution and the increase in chlorophyll accumulation was
compared with incubation in distilled water. Three significant facts
were brought out by the results obtained.

1) In both lines ALA feeding increased the chlorophyll synthesis
by 50% as compared to the control.

2) The mutant synthesized 2/3 the amount of chlorophyll
synthesized by the normal irrespective of incubation with ALA or
distilled water.

3) The incubation with ALA increased the chlorophyll content of the mutant to the level of the normal when it was not incubated with ALA.

From 1 and 2 it could be concluded that the availability of ALA limited accumulation of chlorophyll in both lines in the same proportion. In other words the slower rate of accumulation of chlorophyll in the mutant was not due to a slower rate of ALA synthesis. If the slower rate of ALA synthesis was responsible for the slower rate of chlorophyll accumulation in the mutant then in this experiment the increase in chlorophyll synthesis in the mutant should have been much greater than in the normal and the chlorophyll synthesized by the mutant in presence of ALA should have been equal to the chlorophyll synthesized by the normal. The etiolated seedlings of both the lines were exposed to light for 1 hour prior to incubation, however, the amount of chlorophyll synthesized during this period by both lines was similar. Thus the possibility that the mutant suffered on account of lack of ALA is ruled out and the slow accumulation of chlorophyll in the mutant could not have been due primarily to the enzymes of ALA synthesis being present in limiting amounts. This also confirmed the conclusion drawn earlier that there was no scarcity of building blocks of chlorophylls in the mutant.

If one compares the chlorophyll synthesized by the mutant in the presence of ALA with the normal in the presence of distilled water (table XXIX) it appears that the effect of the mutation has been reversed by ALA. However, both lines synthesized 50% higher chlorophyll in the presence of ALA than in distilled water and the chlorophyll

accumulation in the mutant was only 2/3 that in the normal. There-fore in both lines one control of chlorophyll synthesis was exerted at the synthesis of ALA and a second control was exerted at a later step in the mutant only. On this basis the second control is the one primarily responsible for the reduced chlorophyll synthesis of the mutant. The results suggest that when protein synthesis is inhibited either by application of an inhibitor of protein synthesis or by mutation as in the case of the Gateway mutant under consideration, probably two controls of chlorophyll synthesis are affected at the same time. One of these is at the level of ALA as shown by Gassman and Bogorad (1967a, b) and proposed by others for porphyrin synthesis. The other is the production of holochrome protein as reported by Kirk and Allen (1965). Electron microscopic studies of this mutant by Maclachlan and Zalik (1963) showed that the chloroplasts of the mutant at early stages were not well developed. This together with the results obtained in the present study and by Miller (1965) suggest that the protein responsible for slow accumulation of chlorophyll is the holo-chrome protein.

In addition to yielding information for a comparison of the two barley lines this study provided general information on the changes in free and protein amino acids during seed germination and metabolism of glycine and leucine by 6-day old barley shoots. It was found that during germination the reserve protein of the endosperm was broken down to yield free amino acids which were translocated to the embryo and utilized there for the synthesis of embryonic protein, other nitrogenous compounds and for respiration. The protein of the endosperm

of barley was rich in glutamate and proline which were metabolized during germination to provide for the needs of the embryo.

The metabolism of glycine by 6-day old shoots showed that it was converted to serine, utilized for the synthesis of chlorophyll, protein and other amino acids. A comparison of percent label and specific activity of serine and glutamate in protein and free amino acids suggested the presence of 2 separate pools of both these amino acids in barley shoots. The data on leucine metabolism revealed that this amino acid was metabolized very little and was incorporated into protein directly as leucine. The pattern of metabolism of this acid was essentially the same in light and dark.

The studies on the changes of amino acids during germination, comparison of distribution of label and specific activity of the amino acids in free and protein amino acids in shoots that were metabolizing glycine and leucine clearly indicated that the free amino acids were the immediate precursors of protein.

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Table i. Buffers for amino acid analysis

pH	2.2	3.25	4.25	5.28
Use	Sample dilution buffer	Long column eluent	Long column eluent	Short column eluent
Na Conc.	0.2N	0.2N	0.2N	0.35N
Citric H_2O	21 g	840 g	840 g	491 g
NaOH (97%)	8.4 g	330 g	330 g	288 g
Conc. HCl	16 ml	426 ml	188 ml	136 ml
Caprylic	0.1 ml	4.0 ml	4.0 ml	2.0 ml
Thiodiglycol	20 ml	200 ml	200 ml	--
BRIJ-35 sol.	2.0 ml	80 ml	80 ml	40 ml
Final Volume	1 litre	40 litres	40 litres	20 litres